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13. ABSTRACT ***(Maximum 200 Words)***
Our objective was to investigate the role of integrins in the metastasis of human breast cancer. Integrins play an important role in cell adhesions with extracellular matrix proteins and the maintenance of normal differentiation. Variants of the human breast cancer cell line MDA-MB-435 were selected for different expression of α6 integrin. High-α6 expressing cells showed enhanced binding to laminin and were more metastatic to the lungs of nude mice. MDA-MB-435 cells lack β4 integrin, due to alternate splicing of β4 RNA transcripts. Study of a panel of human breast cancer cells revealed a relationship between α6 expression and malignant growth and metastasis in nude mice. The results support a hypothesis that high α6 integrin expression promotes metastasis of breast cancer cells. A similar study on αvβ3 expression did not find a significant association between this integrin and metastatic ability of MDA-MB-231 breast cancer cells, although a variant of this cell line isolated from a bone metastasis in a mouse had higher expression of this integrin, and bound readily to osteopontin. Modifying expression of osteopontin with antisense constructs in the breast cancer cells altered growth in vitro and in vivo, suggesting that complex interactions may regulate expression of αvβ3 in these cells.

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Janet C Price 9/29/99

PI - Signature Date

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The role of integrins in breast cancer metastasis

Introduction

The subject of this proposal is metastasis, the most common cause of death of women with breast cancer. At the time of diagnosis of breast cancer, prognosis is related to the disease stage and the presence of cancer cells in the axillary lymph nodes. Twenty percent of women with early stage node-negative breast cancer may subsequently develop metastatic disease, while as many as 90% of women with locally advanced breast cancer or with extensive lymph node involvement will have further or more extensive metastasis recurrence. In addition to the axillary lymph nodes, the other common sites of breast cancer metastasis are brain, liver and lungs. However, bone is the most common site of breast cancer metastasis, leading to pain, pathologic fractures, central nervous system compromise and hypercalcemia. The factors determining the pattern of metastasis of breast cancer cells to certain organs, and especially the bone have not been determined.

The objective of this proposal is to investigate the role of cell surface integrins in the metastasis of human breast cancer. Cell surface integrins play an important role in cell adhesions and interactions with extracellular matrix proteins and the maintenance of normal patterns of differentiation. Immunohistochemical analysis of breast cancer specimens has shown that alterations in integrin expression are commonly seen, with either increased, decreased or *de novo* expression of various integrins, or loss of polarization when compared with benign or normal samples. One clinical study showed that increased expression of $\alpha 6$ in primary breast cancers was related to shorter survival(1). In a nude mouse model of breast cancer metastasis developed in our laboratory(2), the cells with highest metastatic capability had higher surface expression of $\alpha 6$ and αv integrin sub-units than poorly metastatic cells. A study using the MDA-MB-435 cell line transfected with a dominant negative variant of the $\beta 4$ integrin subunit, that effectively reduced expression of the $\alpha 6\beta 1$ integrin on the cell surface showed that these cells had reduced invasive capability (3). These results complement our initial observation that the more metastatic cells had higher levels of the integrin expression, and provide further evidence for an association with malignant progression. Breast cancer cells isolated from a nude mouse bone metastasis showed elevated expression of the $\alpha v\beta 3$ integrin. This integrin is also expressed by osteoclasts and mediates binding to the bone matrix protein osteopontin (4). The bone-metastasis derived breast cancer cells bound avidly to the osteopontin, and we propose that elevated expression of the $\alpha v\beta 3$ integrin may mediate events that are critical for the development of bone metastasis.

The scope of our study is to use variants of established human breast cancer cell lines that differ in integrin expression to examine further the relationship between integrin expression and metastasis to different organs. The experimental model is the implantation of the breast cancer cells into immunodeficient nude mice. The experiments are designed to provide new information on the cancer-cell matrix interactions that are part of the metastatic progression of breast cancer. This knowledge could potentially identify new approaches for therapeutic intervention.

Body of Report:

Task 1: Further development of an animal model for human breast cancer metastasis to bone and bone marrow

Experimental method proposed: Injection of cells into the left ventricle of the heart of nude mice is a recognized method of inducing bone metastasis, using various types of cancer cell lines (5; 6; 7). In our preliminary studies, we showed that human breast cancer cells could be recovered from cultures of bone marrow flushed from the femurs of nude mice at intervals after injection into the left-heart.

The original approach was to use genetically tagged cells to examine the kinetics of bone and bone marrow metastasis development, following injection of cells into the left-heart. Tagging cells with the lac-Z gene was proposed. In the original proposal, such cells were to be obtained from another laboratory, but were not made available when requested. Transfection of cells with pCMVβ (from Clontech) was accomplished but expression of the marker gene was not stable or consistent, especially when cells were recovered from metastases in mice. As an alternative, the MDA-MB-231 and the MDA-MB-435 cell lines were transfected with the plasmid pEGFP (Clontech) expressing the green fluorescent protein, that other investigators have shown can be an effective marker in *in vivo* studies (8). In an initial test for retention of the marker gene when the breast cancer cells are injected into nude mice, MDA-MB-231-GFP cells were injected i.v. into nude mice. The numbers and incidence of lung metastases produced by the GFP-transfected cells were very similar to those produced by non-transfected cells. Cultures established from the lung metastases were examined for expression of GFP using a UV microscope, and the marker gene was still expressed. Thus, this method of marking breast cancer cells appears to be stable and appropriate for *in vivo* studies, as reported in the annual report for 1998. However, the original concept of recovering the breast cancer cells from the bone marrow as an indicator of the presence of metastatic cells within the bone and bone marrow has not proven to be a reproducible finding.

Results presented in the Annual Report for 1997 reproduced the original preliminary data presented in the proposal:

Table 1: Detection of breast cancer cells in the bone marrow of nude mice injected into the left-ventricle of the heart

Cell line	Breast cancer cells in marrow	Metastases in other organs
MDA-MB-435	6/8	Lungs, adrenals, brain
MDA-435-BM	6/6	Lungs, adrenals, brain, ovary, mammary fatpad
MDA-MB-231	10/12	Lungs
MDA-MB-468	0/6	Adrenals

The presence of cells in bone marrow was assessed after 10 – 14 days of culture of cells recovered from the femur, and in other organs by macroscopic examination and/or histopathological examination.

When the same experiments were repeated using the MDA-231-GFP cells, the results were inconsistent. In a series of 3 experiments, breast cancer cells were found in cultures from only 3/10 animals injected (the cells expressed GFP under UV illumination) in expt. #1, and in 6/10 cultures in study #2. However, in study #3, no cultures (0/7) were found to contain the breast cancer cells. Histological examination of organs from mice in the last experiment did show the presence of breast cancer cells in the brain and lungs of sampled animals, showing that the injected cells were viable and capable of growth in mice. The conclusion reached is that either the GFP-expressing cells have lost the ability to colonize the bone marrow and bone of nude mice, or that the technique is not suitable for answering the questions we wish to address. Histology of sampled femurs from the mice did not show the presence of breast cancer cells. This bone was chosen, a) based on the preliminary study and Table 1 and b) based on the work of other investigators, who have used the MDA-MB-231 breast cancer cell line and the left-heart route of injection, to consistently produce lytic lesions, which can be found in the femurs of nude mice (7; 9). The femur is easy to image and process for histology.

To test whether the MDA-231 GFP cells had lost the ability to grow in this environment, we injected the cells directly into the tibia of nude mice. Six weeks after injection of the cells, swelling was apparent around the joint, and the mice were killed and the hind limbs examined by X-ray imaging. The Cancer Biology Department has recently acquired a Faxitron MX-20 X-ray unit that is suitable for imaging small objects (prior to this we were unable to image animals, owing to the physical location of radiographic equipment and constraints on transferring animals between different animal housing areas). Figure 1 (Appendix) shows that the MDA-231-GFP cells are capable of forming lytic lesions in the bone of nude mice, when delivered directly into the bone. The tumor lesions were recovered and established in tissue culture, and were found to express GFP. The technique of direct injection, and monitoring the development of lytic lesions with X-ray imaging would be the alternate approach to try and isolate variants of this human breast cancer cell line (and others) that have potential for growth in the bone, which could then be used in further experiments to understand the interactions between breast cancer cells and the bone microenvironment (see also below in Task 4).

As reported in the Annual Report for 1998, an alternative approach was to use a mouse mammary tumor line 66.3, that is highly metastatic to the lungs and lymph nodes of BALB/c mice, as a model for bone and bone marrow metastasis following injection of cells into the left heart. Other investigators using this technique report evidence of bone metastasis from paralysis or visual evidence in the mice injected with a variety of cell lines (5; 6). With the 66.3 cell line the results showed a similar distribution of metastases as we found with human breast cancer cells injected into the left heart of nude mice. The 66.3 cell line is a variant of the same mammary tumor shown in a recent publication to have potential to metastasize to the bone (10). In our experiments, injecting 66.3 cells into the left heart produced a high incidence of tumor nodules in the adrenals, kidney and ovaries of the mice, in addition to the lungs. Histological sectioning of the brains and vertebrae of mice did not show, however, that we could detect the mammary tumor cells in the bone and bone marrow. While cells may be present in the bone and bone marrow, as we believe from previous work of culturing cells from bone marrow of mice injected by this route, the growth of experimental metastases in other organs of the animals (e.g. kidney, adrenals) necessitates euthanasia before growth is manifest in the bone. The cell line used in the report of Lelekakis et al (10) has been acquired by this department, and can be used

in future experiments to develop a murine model of mouse mammary tumor metastasis to bone. As described in the original proposal, the effect of ovariectomy on the development of bone metastases was tested in the 66.3 model. The purpose was to examine whether the bones of mice undergoing resorption due to estrogen removal (11; 12) were more readily colonized by mammary tumor cells. In the Annual Report for 1997, a similar experiment was reported using human breast cancer cells injected into nude mice, three or six weeks after ovariectomy or sham laparotomy. No differences were found in the incidence of bone marrow cultures with breast cancer cells. The study was repeated in immunocompetent mice to try and resolve the possible problem that nude mice do not undergo the same degree of bone resorption, and also that the human breast cancer cells may be less responsive to murine cytokines released by resorbing bone. BALB/c mice were ovariectomized at 5 weeks of age (or sham laparotomy for control) and three weeks later were injected with 2.5 x 10^4 66.3-neo1 cells into the left-heart, i.v. or into the mammary fatpad. The other routes were chosen to test whether the removal of the ovaries would alter the ability of the mammary tumor cells to grow locally and metastasize. The results were identical for all routes of injection, with no differences found in incidence of tumors, lung metastases, or metastases in adrenals, lungs or brain (histology of the brains from left-heart injected mice), whether the mice had the ovaries removed or left intact. Cultures of bone marrow from the femurs of the mice were treated with 400 μg/ml G418 (the 66.3-neo1 cells had been transfected with pSV2neo so that they could be selected using the antibiotic), and showed similar incidence of resistant colonies (2/7 of ovariectomized and 3/9 sham operated mice).

Thus we were unable to show that ovariectomy, which is reported to induce bone resorption in mice altered the ability of breast cancer or mouse mammary tumor cells to grow or survive in the bone or bone marrow of the test animals. Thus, the initial hypothesis that conditions of bone resorption can promote the growth of breast cancer cells may be wrong. Owing to problems mentioned above, the results may be due to technical issues, rather than the biological effects of the resorbing bone environment. As we now have the X-ray imaging facilities available (Fig. 1), the direct injection method could be used to ask the same question, and address the issue of effect of Tamoxifen or a bisphosphonate on the ability of breast cancer cells to survive and grow in the bone environment. Due to the lack of a conclusive result from the ovariectomy experiment, the proposed study of treatment with Tamoxifen and Pamidronate was not performed.

Task 1: Not completed due to technical problems that were not resolved in the course of the period of the award. Data were generated to suggest that an alternate approach, using direct injection of cells into the tibia of nude mice, could be used for a study of the growth of human breast cancer cells in nude mice.

Task 2: Phenotypic characterization of breast cancer cells with different levels of integrin expression

a) Metastatic phenotype of MDA-MB-435 cells expressing different levels of α6 integrin

As reported in the Annual Report 1998, this part is complete and a copy of the galley proofs for the paper is included in the appendix (Mukhopadhyay *et al*, Clinical and Experimental Metastasis, in press, 1999).

b) Isolation of variants expressing different levels of αvβ3 integrin

The rationale for this was the initial observation that a cell line isolated from a bone metastasis of the MDA-MB-231 cell line in nude mice expressed higher αvβ3 levels than the parent line (reported in the poster at the Era of Hope meeting, 1997). The same procedure as used in the Mukhopadhyay et al paper, and described in the Annual Report for 1998, using primary antibody against this integrin heterodimer, LM609, 1:50 dilution. Sterile sorting for individual clones selected from the top 5% of the stained cells were collected and grown in culture. However, when the cells were expanded in culture, and tested for expression, they did not show enhanced expression of the integrin. As reported in the previous report, the antibodies against αvβ3, although from the same clone and manufacturer were less reactive than in the studies presented in 1997 and earlier. Additional antibodies and different dilutions were tested, but no stable increase in αvβ3 expression was found (variants 231-S2,3, and 4, Table 2). An alternate approach was tried, using magnetic Dynatech CELLection Pan Mouse IgG beads. The cells were incubated with antibody to αvβ3 or β3 (1:50 dilution) for 30 min., in serum-free medium, then with the magnetic beads that are bound to anti-mouse IgG. Magnetic separation should enrich the population for cells expressing the antigen of interest. We successfully used this system to enrich populations of MDA-MB-435 that were transfected with a full-length or deleted construct of the β4 integrin. Transfected clones in which 25 – 30% of the cells expressed β4 on the cell surface were subjected to the Dynabead selection process, and resulted in populations with >60% β4 expressing cells. However, as shown below in Table 2, the selection procedure was not successful in isolating variants of MDA-MB-231 with high levels of αvβ3 (variants 231-αvβ3, and β3).

A further attempt was to clone the MDA-MB-231 cell line by limiting dilution (Clones D2, 4, 7, 9, and 20 shown in Table 2), and test a panel of clones for agarose growth ability, and see whether this would produce a panel of clonal variants with different phenotype related to metastatic ability.

Table 2: Integrin expression and agarose colony forming efficiency of MDA-MB-231 clones

Cell line	αvβ3 % positive	MFU	CFE in 0.3% agarose	CFE in 0.6% agarose
MDA-MB-231	14.6%	8.8	49.6%	19.1%
231-S2	0.4%	12.9	20%	4.5%
231-S3	1.2%	7.9	12.5%	1.5%
231-S4	1.5%	5.1	41%	4.2%
231-D2	3.1%	3.9	56.3%	21.8%
231-D4	4.7%	4.3	51.4%	18.3%
231-D7	2.4%	3.4	49.4%	33%
231-D9	3.4%	3.8	50.9%	33%
231-D20	0.3%	5.8	64.4%	35%
231-αvβ3 selected	3.9%	10	nd	nd
231-β3 selected	3.5%	27	nd	nd

Legend for table 2: Percent positive is the percent of cells expressing antigen recognized by the LM609 antibody, at 1: 50 dilution. Cursors for the flow cytometer are set at 2% for isotype matched IgG; MFU = mean fluorescence intensity expressed in relative fluorescent units; CFE, colony forming efficiency = number of colonies > 50μ diameter/number of cells plated x 100.

The results showed that neither of the *in vitro* selection techniques resulted in clones of the MDA-MB-231 with increased αvβ3 expression, but rather these cells expressed less integrin than the original population of cells. The expression studies were repeated using three different antibodies (LM609, Mab 2256 from Centricon, and 7E3, a gift from Centocor, Inc.) with similar results. The growth of cells in semi-solid agarose, and especially growth in ≥ 0.6% agarose has been associated with the ability to grow at metastatic sites in mice (13). As there were some differences between the clones in CFE at 0.6%, some *in vivo* experiments were performed to test the relationship between *in vitro* growth and experimental metastatic ability, using i.v. injection of 1 x 10^6 cells into the tail vein. Mice were killed 7 – 8 weeks later, and the incidence and numbers of lung metastases recorded (Table 3).

Table 3: Experimental lung metastasis of MDA-MB-231 clones

	Expt #1		Expt #2	
Cell line	Incidence	Median # (range)	Incidence	Median # (range)
MDA-MB-231	3/5	13 (0 – 110)	4/5	5 (0 – 25)
231-D4	4/5	40 (0 – 78)	5/5	25 (6 – 110)
231-D20	1/5	0 (0 – 24)	5/5	35 (18 – 77)
231-D2	nd	-	4/5	22 (0 – 120)
231-D9	nd	-	4/5	60 (0 – 73)

Incidence = number of mice with lung metastases/number of mice injected.

Two outcomes were apparent from these data, from admittedly small groups of mice. One is that there was not good agreement between experiment #1 and #2 for 231-D20 cells, which initially appeared to be poorly metastatic, yet in the second experiment produced numerous metastases in all mice injected. Second, that the ability to form lung colonies was similar in the 4 clones, two which had the same CFE in 0.6% agarose as the parental cells, and two that showed an almost two-fold higher CFE in agarose. Thus, the *in vitro* assay is not a good predictor of *in vivo* growth ability of clones of this breast cancer cell line. Comparing the lung metastasis data with the αvβ expression data shown in Table 2, there is no support for the initial hypothesis that elevated levels of this integrin would promote the metastatic phenotype of the breast cancer cells, at least in the lung colonization assay we used. Our findings do not therefore support those published by Wong et al, (14), showing higher αv expression in human breast cancer cells with invasive and malignant properties. Possibly the higher αvβ3 expression found in the initial studies is a characteristic of breast cancer cells with ability to grow in the bone, rather than metastasis to other organs. Human breast cancer cells in pathological specimens of bone metastases have been reported to show a high incidence of αvβ3 expression (15).

The inability to isolate clones with higher αvβ3-expression was difficult to explain, given that we successfully used the same approach with the α6 integrin project. This led us to consider that the expression of this integrin is regulated by some additional, unknown factors. In a related study that came about from work on osteopontin (OPN) interactions showed that β3 expression in the cells may be related to the levels of OPN expressed. In a manuscript included in the appendix, Mukhopadhyay and Price, "Differential effects of osteopontin antisense RNA on the growth of human breast cancer cells in vitro and in vivo", we report these findings. The preliminary observation that led to this study came from phenotypic characterization of the MDA-231 bone metastasis derived cells. In the annual report of 1997 (and summarized in Figure 2 in the appendix), we noted that the bone metastasis derived cells bound readily to OPN, which is a component of the bone matrix. The binding could be blocked by antibodies that antagonized the αvβ3 integrin (Fig 2 b and c). As it has been reported that breast cancers express OPN (16), we performed northern blot analyses, and determined that variants from metastases of MDA-MB-231 in lungs or bone of nude mice expressed higher levels of OPN (Fig.3). An antisense OPN construct was made into pRc/CMV and transfected into lung-metastasis derived MDA-231-LC1 cells. Stable clones were generated after neomycin selection. Antisense RNA inhibited 50 – 60% of endogenous OPN RNA and protein, as measured by RT-PCR and western blot analyses. In cell proliferation assays, the antisense-transfectants showed 50 – 60% reduced growth rates in colony formation assays, and were more sensitivity to etoposide and staurosporine. In addition, treatment of these cells with an antibody that recognizes the αvβ3 integrin partially inhibited growth, implying that interaction between OPN and this integrin may regulate growth and survival of these cells. The clones with reduced OPN expression formed tumors in the mammary fatpad of nude mice at a similar frequency as the control cells, yet the potential for experimental metastasis to the lungs of nude mice was increased. Our results suggest the function of OPN in malignant progression of breast cancer cells is complex and may depend upon the growth environment. Similarly, the expression of the αvβ3 integrin receptor of OPN may be subject to regulation by autocrine or paracrine factors, which confounded attempts to isolate by *in vitro* techniques clones that expressed consistently different levels of the integrin.

Task 2:a) α6 integrin study completed, and data presented to support the hypothesis that α6β1 expression is associated with high metastatic capability of human breast cancer cells.
b) αvβ3 study – the data generated did not support a hypothesis that this integrin is associated with metastatic capability of breast cancer cells. Data from a study to test the role of OPN of MDA-MB-231 metastasis suggested a co-regulation of OPN and the β3 subunit of the integrin, pointing to a more complex regulation of the integrin expression than originally anticipated.

A final report describing the characterization of the MDA-MB-231 bone metastasis derived cells, and the OPN binding and expression is being completed, and will include some new data to be discussed under Task 4.

Task 3: Characterization of integrin profiles of human breast cancer cells

This Task originally had been limited to a study of the variants derived from metastases in nude mice, yet was extended to include a panel of human breast cancer cell lines with a range

of tumorigenic and metastatic capabilities. The results were presented in the Annual Report 1998, and are part of the publication referenced above (Mukhopadhyay *et al.*, in press 1999). One novel observation was the identification of alternate splicing in the β4 integrin in the MDA-MB-435 breast cancer cell line. As the α6 integrin heterodimerizes with β4 or β1, we have initiated studies in which the full-length β4 integrin has been introduced into MDA-MB-435 clones with different levels of α6 expression. Our objective is to compare how the different heterodimers, with distinct intracellular signaling pathways (17-19) may influence the tumorigenic and metastatic properties of the breast cancer cells.

Further analyses cells with different αvβ3 expression, which was to form part of this task was not continued, owing to the results found in Task 2 (described above). Expression of focal adhesion kinase, $p125^{FAK}$, was studied in some of the clones and variants, using immunoblotting and immunoprecipitation. Fig. 4 in the appendix shows the $p125^{FAK}$ levels in cells with different levels of OPN expression (clones #1, 3 and 5 express less endogenous OPN, and have lower αvβ3 expression). No difference could be detected in $p125^{FAK}$ expression or activation (measured by blotting with PY-20 antibody against phosphorylated tyrosine), in the MDA-MB-231 clones, when cultured on either plastic, or on collagen or Matrigel coated plates. The initial premise that we might see differences was probably over-simplistic, considering the variety of integrins expressed by the breast cancer cells that can interact and activate with this kinase (20).

Task 4: To investigate whether cytokines modulate integrin expression in metastatic breast cancer cells

Part a) of this task compared the integrin expression in MDA-MB-231 and bone metastasis derived cells following exposure to IL-6, at doses (100 or 500 ng/ml recombinant human-IL-6) which did not alter the growth of the cells *in vitro*. Cells were harvested for analysis of integrin expression after 72h exposure to IL-6, and flow cytometry performed as described in the articles included in the appendix (Table 4).

Table 4: Integrin expression following treatment with IL-6

Cell line/treatment	αvβ3		αv		α6	
	%	MFU	%	MFU	%	MFU
MDA-231/control	62.3	1.4	98	5.4	99	17.2
MDA-231/100ng/ml IL-6	67.2	1.8	97.3	5.2	100	17
MDA-231/500 ng/ml IL-6	64.4	1.6	99	5.4	100	18.1
231-bonemet/control	92	4.8	99	12	99.6	11
231-bonemet/100ng/ml IL-6	92.9	4.3	99	10.2	99	10.8
231-bonemet/500ng/ml IL-6	90.6	4.4	99	10	99.3	10.2

% = percent positive cells; MFU = mean fluorescence in relative fluorescent units.

Thus no alteration in integrin expression was apparent following exposure of the cells to IL-6. This cytokine had been reported to promote the motility of breast cancer cells, and alter the cell-cell attachments (21), but did not alter the protein expression of αv, αvβ3 or α6 integrins in MDA-MB-231 breast cancer cells. Similar findings were obtained with treatment of the cells

with TGF-β1 (0.5 – 50ng/ml). As we could not show changes in the integrin expression, the next step of testing whether the same cytokine altered binding to extracellular matrix protein was not performed. If altered binding had been noted, the investigation would require changing focus to other adhesion molecules, since the integrins of interest in the original proposal did not show altered expression in the MDA-MB-231 cell line.

Since submitting the proposal, some additional information was presented in the scientific literature showing that some of the cytokines present in resorbing bone (IL-6, IL-11, TGF-β1) are expressed by the breast cancer cells (9; 22; 23). The levels of expression of these three cytokines, and parathyroid hormone-related protein (PTHrP) (24; 25), another known activator of osteoclast activity, were determined in the metastasis-derived variants of MDA-MB-231 (Figure 5). The RT-PCR analyses, showing transcript levels of the cytokine and either actin or GAPDH as a reference transcript (amplification using both sets of primers in the same reaction mixtures), indicated that the variants of MDA-MB-231 express similar levels of TGF-β1 and IL-11 transcripts, yet the metastasis derived cells have higher levels of IL-6 and PTHrP. The results from ELISA assays for IL-11 released into culture supernatants by the breast cancer cells reflected these results. The study was extended to further breast cancer cell lines (GI101A, MDA-MB-361, MCF-7, MDA-MB-435) and to samples from short-term cultures of surgical specimens of bone metastases from a breast cancer patient (BBM-1) and a renal cell cancer patient (RBM-1). RT-PCR for the same cytokines was performed and results are shown in Figure 6. The initial results show heterogeneity of expression of these factors. PTHrP was expressed by the bone metastasis cultures, and by two of the breast cancer cell lines (MDA-MB-361, GI101), with barely detectable levels in the MDA-231 and 435 cells (although see Fig. 5 for another assay). IL-11 transcripts were amplified in the MDA-MB-231 sample, with a weaker signal from GI101 and BBM-1. The renal cell cancer bone metastasis, RB-1, however showed a strong signal for IL-6. Further studies are planned to test whether the high PTHrP-expressing breast cancer cells can grow in the bone of nude mice, following direct injection (see Fig. 1). In addition, the bone-metastasis derived cancer cells will be used for further analyses, such as integrin expression, to extend the observations made with the established cell line, MDA-MB-231. There is to our knowledge only one breast cancer cell line reported in the literature that was established from a bone metastasis (26); most established cell lines originated from pleural effusions. If we can continue to collect and establish short-term cultures from bone metastases from breast cancer (and other types of cancer) patients, we can generate more data on the phenotype of cell that are able to grow in the bone. This will include the osteolytic cytokines, and integrins αv and β3.

Our preliminary findings support those of others, that breast cancer cells express IL-6, IL-11, TGF-β1 and PTHrP, which can stimulate further cytokine release from osteoblastic cells, which in turn may lead to activation of osteoclasts and thus contribute to bone destruction around a metastasis(25). The majority of breast cancer bone metastases are lytic lesions.

Task 4:Initial data showed that exposing MDA-MB-231 breast cancer cells to IL-6 and TGF-β1 did not alter integrin expression. The second part of this task was not performed, since the rationale for the study was to test how the altered integrin expression would relate to binding to matrix proteins. We found that the breast cancer cells themselves express IL-6, IL-11, TGF-β1 and PTHrP at variable levels (by RT-PCR), and that the bone-metastasis derived MDA-MB-231 cells showed elevated transcripts of PTHrP and IL-6, with unchanged TGF-β1 and IL-11

expression. An investigation of the characteristics of bone metastatic cells will continue with these cytokines and osteolytic factors, and the expression of αv and β3.

Key Research Accomplishments

1. Demonstration of correlation between α6 integrin expression and tumorigenic and metastatic properties of human breast cancer cell.
2. Identification of a novel RNA variant of the β4 integrin in human breast cancer cells, which lead to abrogation of protein expression.
3. Showing differential effect of osteopontin expression on the in vitro and in vivo growth of human breast cancer cells.
4. Characterization of bone-metastasis derived variant of a human breast cancer cell line, with elevated expression of αvβ3 integrin and of two osteolytic factors, IL-6 and PTHrP.

Reportable Outcomes:

Manuscripts:
Mukhopahdyay et al, in press, *Clinical and Experimental Metastasis* , "Increased levels of α6 integrins are associated with the metastatic phenotype of human breast cancer cells".
Mukhopadhyay and Price, submitted for publication, *International Journal of Oncology,* "Differential effects of osteopontin antisense RNA on the growth of human breast cancer cells in vitro and in vivo".

Abstracts of presentations at international cancer research meetings:
Price et al., "Increased vitronectin receptor expression by breast cancer cells selected for growth in the bone of nude mice", presented at the Era of Hope Breast Cancer Research Program Meeting, November, 1997.
Mukhopadhyay, R. and Price, J.E., "Expression of alpha 6, beta1 and beta 4 integrins in human breast cancer cell lines", presented at the Annual Meeting of the American Association for Cancer Research, March 1998.
Mukhopadhyay, R. and Price, J.E., "Expression of alpha 6, beta1 and beta 4 integrins in human breast cancer cell lines", presented at the VII International Congress of the Metastasis Research Society, October, 1998.
Mukhopadhyay, R. and Price, J.E., "Stable expression of antisense osteopontin inhibits the growth of human breast cancer cells" presented at the Annual Meeting of the American Association for Cancer Research, April, 1999. Dr Mukhopadhyay received a AACR-Janssen Research Foundation Young Investigator Award for this abstract.

Presentation:
Price, J.E. "Microenvironmental factors in breast cancer metastasis to bone", presented in the UT M.D. Anderson Cancer Center Institutional Grand Rounds, January 1999.

Cell lines:
Isolated clones of MDA-MB-435 breast cancer cell line with different levels of α6 integrin and metastatic potential in nude mice.

Isolated stable transfectants of MDA-MB-231 with altered levels of osteopontin expression and increased metastatic ability in the lungs of nude mice.
Transfected the green fluorescent protein (GFP) vector into MDA-MB-231 cells and deriving a stable clone, which retain metastatic capability to the lungs of nude mice.

Conclusions

The aim of further development of a reliable model of breast cancer metastasis to bone using left-heart injection of cells into nude mice was not achieved, possibly due to technical problems that were not resolved within the time of the award. The results obtained did not support the hypothesis that breast cancer or mammary tumor cells showed altered ability to survive and grow in the bone marrow of mice following ovariectomy, as a stimulus of bone resorption. An alternate approach for an experimental model for answering questions about what phenotypes are characteristic of bone metastatic-breast cancer cells has been identified. Direct injection of breast cancer cells into the nude mouse bone and recovery of cells from bone lesions may generate selected populations of cells, that will give more information on an aspect of breast cancer metastasis that is a significant clinical problem. A high percentage of women with metastatic breast cancer develop bone metastases, yet there is limited information about the interactions of breast cancer cells with the bone environment. The studies with the human breast cancer cells and the nude mouse model, together with similar studies on cells recovered from surgical specimens of bone metastases, may provide some of this information. Based on this knowledge, new approaches could be taken to restrict the development and growth of breast cancer bone metastases.

High expression of the $\alpha 6$ integrin was significantly associated with the tumorigenic and metastatic properties of human breast cancer cells. The most metastatic cells in the panel studies expressed high levels of the $\alpha 6\beta 1$ heterodimer. No $\beta 4$ integrin protein, the alternate β-unit that dimerizes with $\alpha 6$, was found in these cells, due to a novel RNA splicing event that abrogated protein expression. More information about the regulation of expression of $\alpha 6$ integrin in different breast cancer cell lines can potentially direct further studies of how to reduce expression, and hence suppress the metastatic phenotype. Similar studies of the $\alpha v\beta 3$ integrin did not support the hypothesis that elevated expression of this cell surface molecule was significantly associated with metastasis in the model used. Breast cancer is a heterogeneous entity, and one limitation of the models we use for experimental studies is that individual cell lines may not be good representatives of the disease. More information about the regulation of expression of different adhesion molecules, such as the integrins studied in this proposal, is needed to better understand how breast cancer cells form metastases in different organ environments.

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Personnel receiving pay from the research effort:

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Ratna Mukhopadhyay, Ph.D., postdoctoral fellow

Galina Kiriakova, B.S., Rsearch Assistant I.

Final report for Grant Number DAMD17-96-6224

The role of integrins in breast cancer metastasis

Appendices:

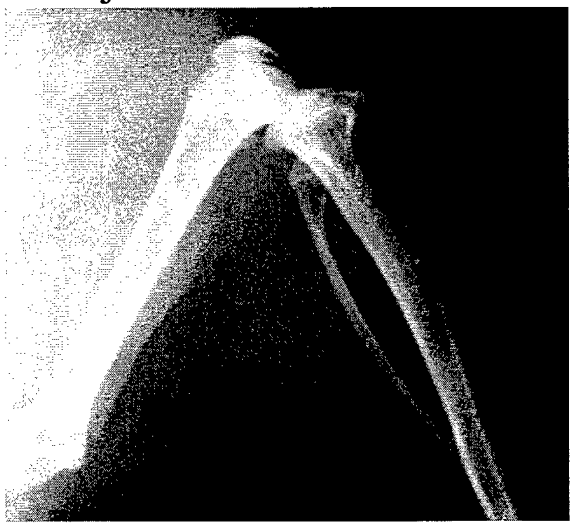
Figures 1-6
Figure legends

Galley proof: Mukhopadhyay et al.
Manuscript: Mukhopadhyay and Price

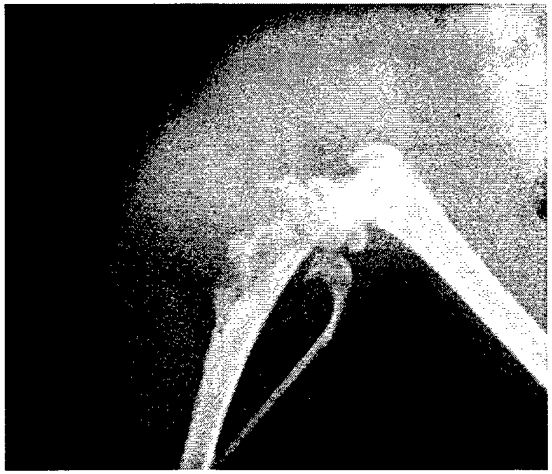
Figure 1

X-ray images of nude mice injected with human prostate cancer (PC3mm2) or human breast cancer (MDA-231 GFP) cells. No injection image shown for reference of normal appearance of bones.

No injection



PC3mm2 (4 weeks post injection)



MDA-231-GFP (6 weeks post injection)

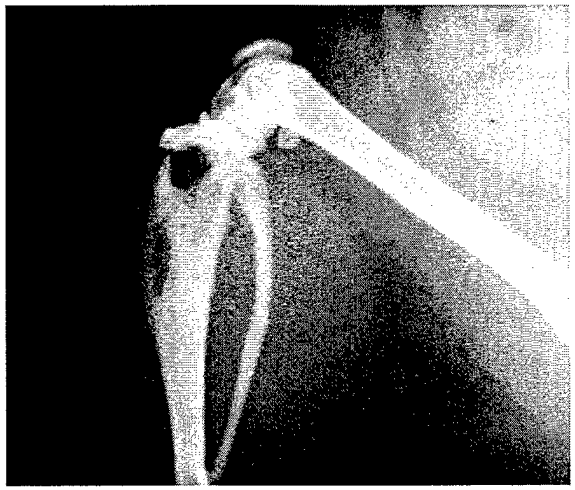
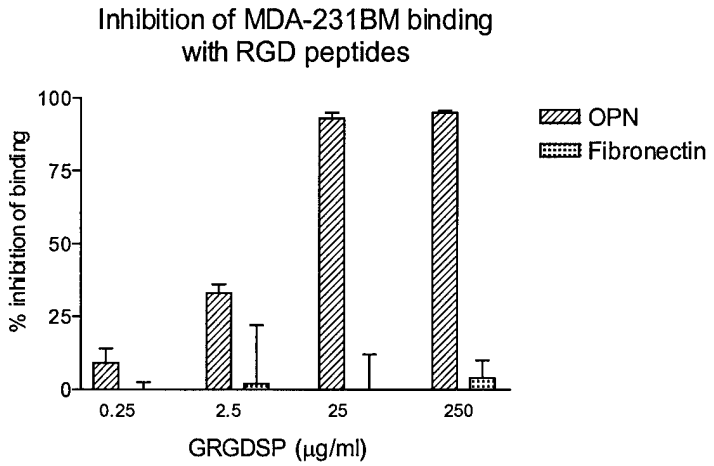
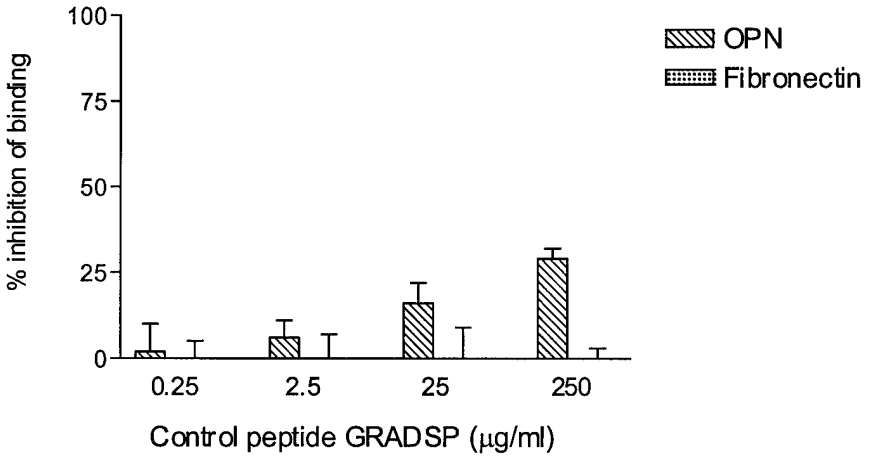


Figure 2


Inhibition of MDA-231BM binding
with RGD peptides
% inhibition of binding
100
75
50
25
0
0.25
2.5
25
250
GRGDSP (μg/ml)
OPN
Fibronectin


% inhibition of binding
100
75
50
25
0
0.25
2.5
25
250
Control peptide GRADSP (μg/ml)
OPN
Fibronectin

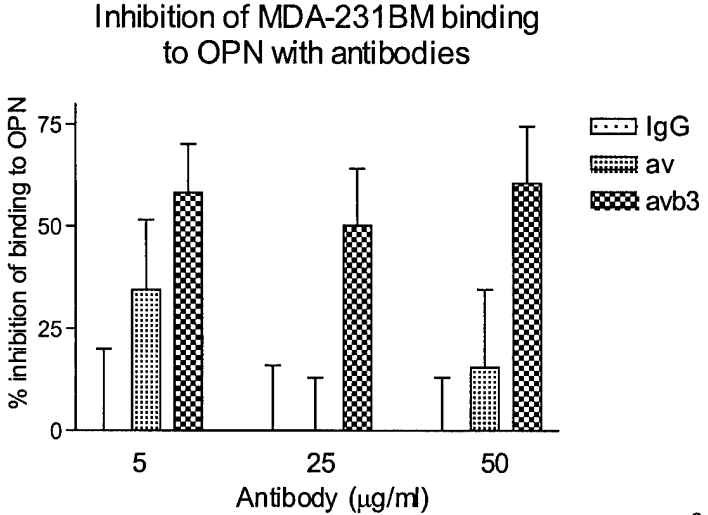

Inhibition of MDA-231BM binding
to OPN with antibodies
% inhibition of binding to OPN
75
50
25
0
5
25
50
Antibody (μg/ml)
IgG
av
avb3

FIGURE 3

Osteopontin expression in breast cancer cell lines
28S
18S
MDA-MB-435
MDA-435-Lung2
MDA-435 Br1
MDA-MB-231
MDA-231 bone met
MDA-231 Lung1
MDA-231 Lung 2
SAOS-2

Figure 4: Expression of Focal Adhesion Kinase

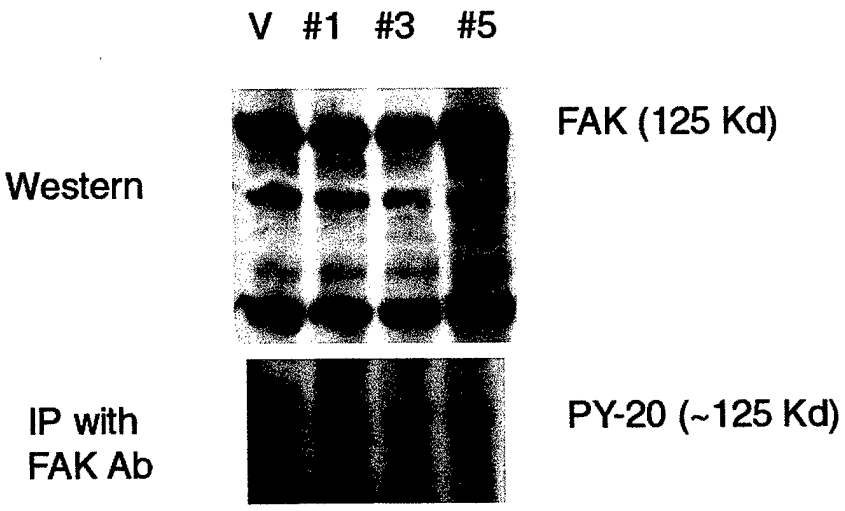


Figure 5:

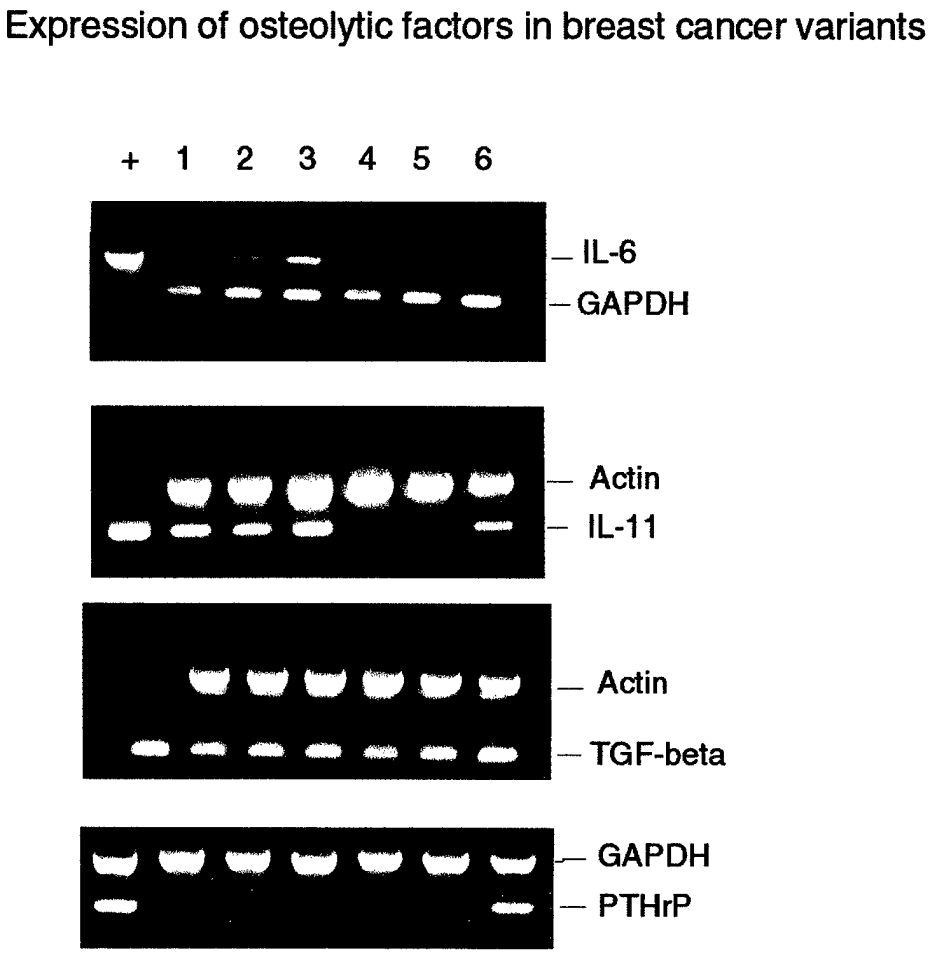

Expression of osteolytic factors in breast cancer variants
+ 1 2 3 4 5 6
IL-6
GAPDH
Actin
IL-11
Actin
TGF-beta
GAPDH
PTHrP

Figure 6:

RT-PCR of Osteolytic Factors expressed by Breast Cancer Cells

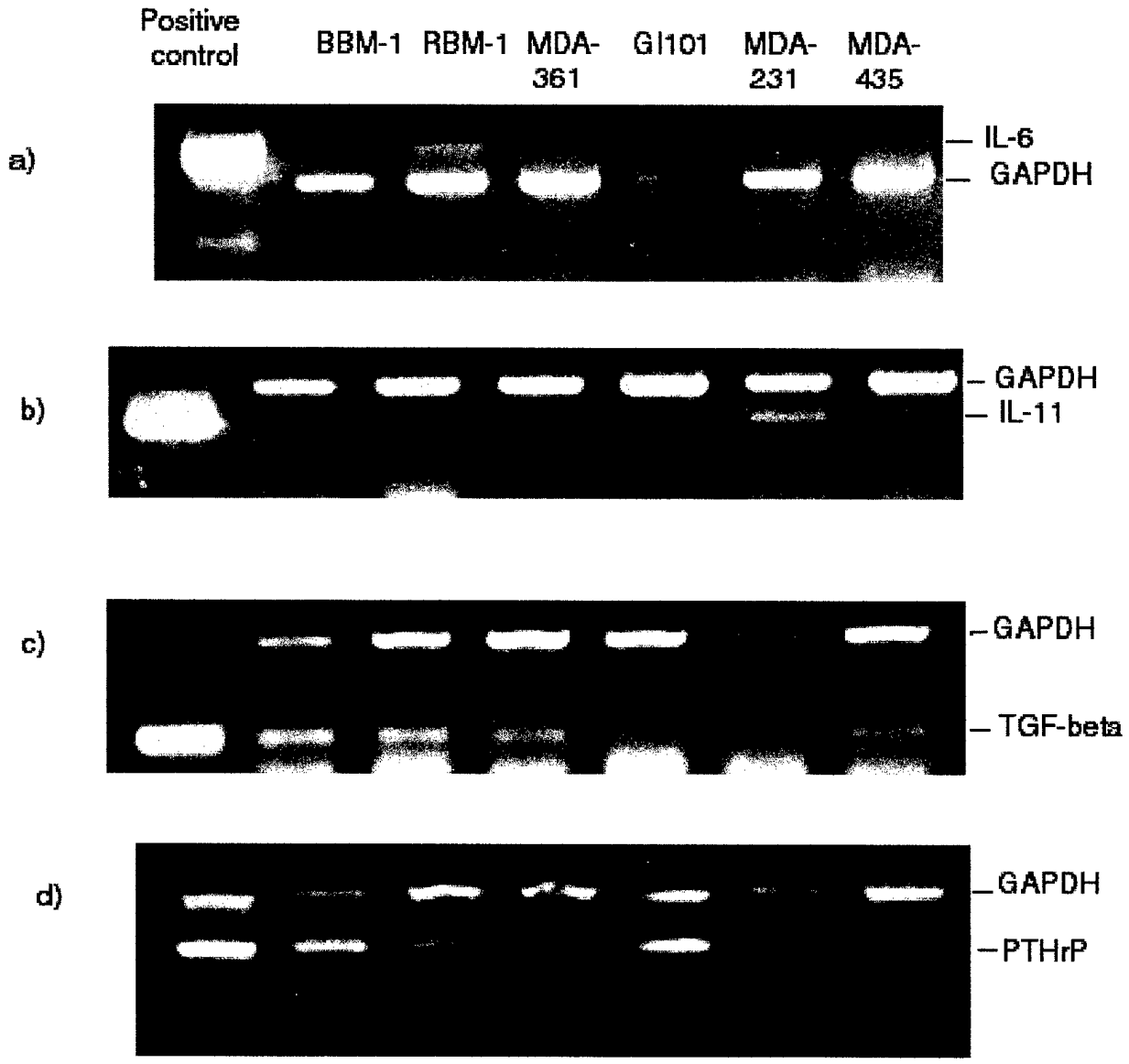


Figure legends:

Figure 1: X-ray images of nude mice injected into the tibia with human prostate cancer cells (PC3mm2) or human breast cancer cells (MDA-231 GFP). Tumor growth in both cases is associated with bone destruction, with clear zones of lysis in the bone at site of breast cancer growth. A normal mouse tibia is included for reference (no injection).

Figure 2: 2a: Inhibition of binding of MDA-231BM cells to OPN in the presence of GRGDSP. Cells were incubated with or without the peptide for 30 min., then plated onto OPN-coated microtiter plates. After 60 minutes incubation, non-attached cells were washed off, and relative numbers of bound cells determined with the MTT assay. The results are expressed as % inhibition of binding, compared with binding to OPN in the absence of peptide.
2b: Identical experiment, using a control peptide, GRADSP, that lacks the RGD sequence.
2c: Inhibition of binding of MDA-231BM cells to OPN, following incubation of cells with monoclonal antibody to αv or αvβ3 integrin or mouse IgG, at the concentrations shown. Binding was compared with binding to OPN in the absence of antibody or IgG.

Figure 3: Northern blot analysis of osteopontin expression in human breast cancer cell lines. Total RNA was extracted from cell cultures and separated in a 1.2% agarose gel using MOPS/formaldehyde buffer. RNA was transferred to a membrane and hybridized with a full length OPN probe labeled with 32-P by random priming.

Figure 4: Expression of Focal Adhesion Kinase (p125-FAK) in MDA-MB-231 variants expressing different levels of osteopontin (V = control, #1, #3, #5 are antisense clones, see Mukhopadhyay and Price, ms. attached). Lysates of cells were immunoprecipitated with antibody to p125-FAK, and blotted for protein levels, and with antibody PY-20 to phosphorylated tyrosine. No differences were detected in levels or activation of the focal adhesion kinase.

Figure 5: Expression of osteolytic factors in breast cancer variants. RT-PCR was performed on cDNA samples from breast cancer cells, using primers recognizing human IL-6, IL-11, TGF-β1 and PTHrP. Primers for either GAPDH or β-actin were included in the reaction mixtures. Amplification products were separated on agarose gels, stained with ethidium bromide and visualized under UV illumination. Legend: +, positive control provided with primers for IL-6, IL-11 or TGF-β1 by Clontech; or cDNA from the squamous cell cancer Colo-16 as positive control for PTHrP; lane 1 = MDA-MB-231; lane 2 = MDA-231-LC1, from a nude mouse lung metastasis; lane 3 = MDA-231 bone met, from a nude mouse bone metastasis; lane 4 = MDA-MB-435; lane 5 = MF-7; lane 6 = SAOS osteosarcoma (cells that retain some properties of osteoblastic cells).

Figure 6: Expression of osteolytic factors expressed by breast cancer cells. RT-PCR was performed as in Fig. 5. BBM-1 and RBM-1 are samples from cultures from bone metastases in patients with breast cancer and renal cell cancer, respectively. The other samples are established human breast cancer cell lines.

Clinical & Experimental Metastasis **0:** 1–8, 1999.

Increased levels of $\alpha 6$ integrins are associated with the metastatic phenotype of human breast cancer cells[1]

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Key words: breast cancer, integrins, metastasis, progression, xenograft model

Abstract

Integrins play an important role in interactions between cells and the extracellular matrix, and thus have a potential role in metastasis. Expression levels of $\alpha 6$, $\beta 1$ and $\beta 4$ integrin sub-units were measured in a panel of human breast cancer cell lines by RT/PCR, immunoprecipitation and flow cytometry. All the lines expressed $\alpha 6$, with the highest levels in the MDA-MB-231 and MDA-MB-435 cells. These grew the most aggressively and were metastastic in nude mice. Low levels of $\alpha 6$ protein were measured in breast cancer cells that were poorly tumorigenic and non-metastatic in nude mice, and there was an inverse relationship between ER and $\alpha 6$ expression. RT/PCR revealed that all lines expressed the 2 isoforms of $\alpha 6$, with the $\alpha 6$A isoform generally more abundant than $\alpha 6$B isoform. Clones of MDA-MB-435 were isolated by sterile sorting for cells with high or low $\alpha 6$ expression, and two variants established from metastases in nude mice were found to differ in $\alpha 6$ expression. When injected into nude mice, the $\alpha 6$-high variants produced significantly more lung metastases than the $\alpha 6$-low variants. $\beta 1$ was abundant in all lines, while $\beta 4$ was not detected in MDA-MB-134 cells, and in the MDA-MB-435 cells an alternately spliced variant of $\beta 4$ was identified. Sequencing of the alternate variant revealed a novel sequence from a splicing event in the cytoplasmic tail of $\beta 4$. None of the cells with this variant mRNA expressed detectable levels of $\beta 4$ protein. Our results suggest that high $\alpha 6$ expression in human breast cancer cells is associated with tumorigenicity and metastatic potential.

Abbreviations: EMT – epithelial-mesenchymal transition; ER – estrogen receptor; mfp – mmary fatpad; RT-PCR – reverse transcriptase-PCR.

Introduction

Integrins are heterodimeric, transmembrane glycoproteins that are formed by the non-covalent association of α and β sub-units. As transmembrane proteins, they can interact with extracellular molecules and intracellular proteins, and these interactions define their functions. Integrins can mediate binding to various substrates, including extracellular matrix proteins, other cell surface molecules, and in some cases to other integrins. The cytoplasmic domains of integrin sub-units can engage various intracellular proteins that link up with the cytoskeleton or with adapter proteins that have known signalling functions [1–3]. At all stages in the metastatic process cancer cells interact with their immediate environment via soluble factors and contact with other cells and extracellular matrices. Altered expression of integrins mediating such interactions have been postulated to be involved in malignant cell growth and in the process of metastasis [4, 5]. There are a number of examples showing how introducing or upregulating integrins can increase the metastatic potential of cancer cells [6–8]. Conversely, blocking integrin expression or function using antisense constructs [9], antibodies [10], or with competing RGD peptides can reduce metastatic potency [11].

The $\alpha 6$ integrin sub-unit dimerizes with $\beta 1$ or $\beta 4$ to form receptors that bind to laminin. The $\alpha 6\beta 4$ heterodimer forms a component of hemidesmosomes and is important for the organization and maintenance of epithelial structures [2, 12]. Mice lacking either $\alpha 6$ or $\beta 4$ sub-units share the same phenotype of abherrant and blistered epithelium [13]. Alterations in the expression of different integrins in malignant breast cells have been reported, with examples of increased, decreased, or inappropriate protein expression [14–17]. Higher expression of $\alpha 6$ has been correlated with shorter survival in women with breast cancer. In the study by Freidrichs et al. [18] 88% of the breast cancer cases with distant metastases had high $\alpha 6$ expression in the primary tumor.

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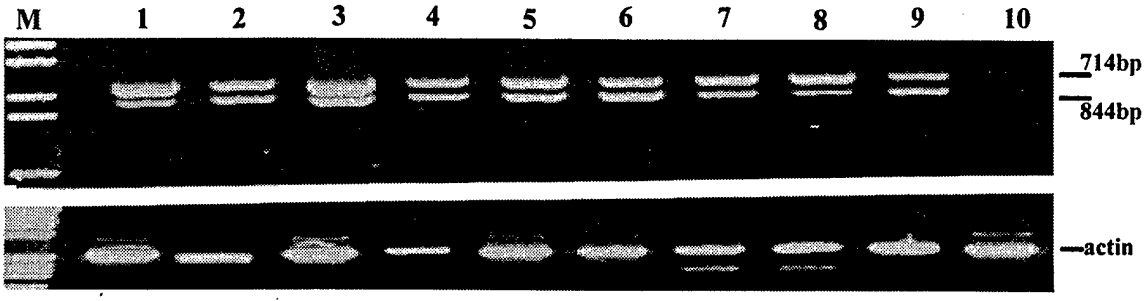

Figure 1. RT-PCR of α6A (844 bp product) and α6B (714 bp product) isoforms from cDNA of human breast cancer cells. Legend: M, marker; lane 1, HBL-100; lane 2, MDA-MB-134; lane 3, MDA-MB-231; lane 4, MDA-MB-361; lane 5, MDA-MB-435; lane 6, MDA-435Lung2; lane 7, MDA-MB-468; lane 8, BT-20; lane 9, BT-474; lane 10, MCF-7

Another clinical study examining both α6 and β4 expression in breast cancer biopsies also linked higher α6 expression with a greater risk of metastasis [19]. This study also reported that expression of laminin in conjunction with the integrins was indicative of poor prognosis, an observation reinforcing the concept of disordered expression of integrins and matrix by cancer cells.

In this study we sought to test the hypothesis that higher α6 expression was associated with increased malignant potential of breast cancer cells. We evaluated the expression levels of α6, β1 and β4 integrins in a panel of human breast cancer cell lines, using RT/PCR and protein measurements. The results showed that expression of these integrins is regulated at both mRNA and protein levels, and that α6 expression is inversely related to ER expression. Furthermore, that elevated levels of the α6 integrin were associated with a more aggressive malignant behavior and metastasis of MDA-MB-435 breast cancer cells, as assessed in a nude mouse model.

Materials and methods

Cell Culture

Human breast cancer cell lines were cultured in Eagle's Minimum Essential Medium supplemented with 5% fetal bovine serum (FBS), nonessential amino acids, L-glutamine, sodium pyruvate, and vitamins (GIBCO-BRL, Grand Island, NY); the supplemented medium was termed CMEM. Cell lines were maintained in monolayer culture in a humidified 37 °C incubator with a 5% CO_2–95% air atmosphere.

Flow cytometric analysis

Cells grown to 70–80% confluence were harvested with trypsin and EDTA and suspended in PBS with 1% FBS at 1×10^6 cells per ml. Monoclonal antibodies against α6 (clone GoH3) or β4 (clone 439-9B) (Pharmingen, San Diego, CA) were added at a 1:500 dilution and incubated at 4 °C for 30 min. The cells were then washed with PBS containing 2% FBS and 0.02% sodium azide and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (Sigma Chemical Co., St Louis, MO) at a dilution of 1:20 at 4 °C for 30 min. The cells were washed extensively in PBS and fixed in 1% paraformaldehyde in PBS. Flow cytometry was performed on an EPICS Profile Cell Sorter (Coulter, Hialeah, FL) with a 525-nm band pass filter to detect FITC and gated on forward versus side scatter to exclude debris, dead cells, and clumps. Analysis was based upon cursors set at 2% for isotype-matched negative controls. For sterile sorting of the MDA-MB-435 cell line, 5×10^6 cells were incubated with the α6 antibody and FITC-anti rat IgG as described above, using wash buffers without sodium azide, and the cells were resuspended after the final wash in sterile PBS. Sorting was performed on a EPICS Elite Analyzer, and approximately 5×10^5 cells sorted into α6-high and α6-low groups. Single cells from each group were plated into individual wells of a 96-well microtiter plate, and resulting clones expanded for subsequent studies.

Cell surface biotinylation and immunoprecipitation

Cell cultures were incubated with 0.5 mg ml^{-1} NHSLC-Biotin (Pierce, Rockford, IL) in HEPES buffer for 30 min at 4 °C, rinsed with HEPES buffer containing protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN), and the cells collected by scraping. Cell pellets were obtained by centrifugation at 500 *g* for 5 min, then solubilized in 200 μl of the same buffer with occasional vortexing. Insoluble material was removed by centrifugation at 10 000 *g* for 5 min. Aliquots of lysates with equal amounts of total protein were incubated overnight at 4 °C with an excess of monoclonal antibody. Immune complexes were immunoprecipitated for 1 h with 20 μl of protein A/protein G agarose (Oncogene Research Products, Cambridge MA) The bound complexes were washed and separated on 7.5% SDS-PAGE gels under reducing conditions, and transferred to nitrocellulose filters. Biotin-labeled proteins were detected by incubating the filters with horseradish peroxidase-linked streptavidin (Pierce), and ECL Western blotting detection reagents (Amersham Life Science Ltd., Little Chalfont, UK), following the manufacturers' instructions.

RNA extraction and RT-PCR analysis

Total RNA from the breast cancer cell lines was isolated using TRI-reagent (Sigma Chemical Co). cDNA was synthesized from 5 μg of total RNA using oligo dT as primer, then used to amplify α6, β1 or β4 PCR products with 1 μM each of the 5′ and 3′ primers,

200 μM dNTPs and *Taq* DNA polymerase (Promega, Madison, WI). The oligonucleotides were obtained from Genosys (The Woodlands, TX). The primers for α6 mRNA were; sense 5′-CTAACGGAGTCTCACAACTC-3′; antisense 5′-ACTCTGAAATCAGTCCTCAG-3′, nt 2656-3499 [20]. The β1 primers were; sense 5′-CAAGGTAGAAAGTCGGGACA-3′; antisense 5′-CACAGT TGTTACGGCACTCT-3′, nt 2116-2454 [21]. Two sets of β4 primers were used; Sense#1 5′-CTCAGAACACTCACAC TCGA-3′; antisense#2 5′-GAGATGTGGGCCCCAGGGAG-3′, nt 4679-4820; Sense#2 5′-GACGGCGGCGCGGCGGGA AGGGCGGCAGCCGTGCCCCAG-3′; antisense #2 5′-AGCTCACACTCACAAGACTC-3′, nt 4441-4679 [22]. Primers for β-actin were; sense 5′-GTGGGGCGCCCCAGG CACCA-3′, antisense 5′-CTCCTTAATGTCACGCACGATT TC-3′. The amplified PCR products were size fractionated by electrophoresis in 4% Nusieve agarose (FMC Bioproducts, Rockland, ME) and in some instances in 2% agarose gels. The gels were stained with ethidium bromide and visualized and photographed using a UV transilluminator. PCR products were quantified by densitometric analysis and normalized with actin. The sequences of the amplified PCR products for selected samples were confirmed by DNA sequencing. Spliced variants of β4 from the MDA-MB-435 cell line was cloned into TA vector and sequenced.

Tumorigenicity and metastasis assays in nude mice

The *in vivo* malignant properties of the human breast cancer cell lines were asessed by injection into nude mice. Tumorigenicity was evaluated by injection into the mammary fatpad (mfp), as described previously [23]. In brief, a small incision was made in the skin over the thoracic fatpad of anesthetized female mice and the mfp exposed. Tumor cells in a volume of 0.1 ml were injected into the mfp, and the incision closed with wound clips. In certain experiments, a 60-day release pellet of 0.72 mg 17-β estradiol (Innovative Research of America, Sarasota, FL) was implanted s.c. at the time of tumor cell injection. Tumor growth was monitored by weekly measurements using calipers, and tumors of 1.5 cm mean diameter removed surgically. Mice were killed 4–6 weeks after tumor resection. The experimental metastatic potential was assessed by i.v. injection of 10^6 cells and mice were killed up to 90 days later.

Laminin binding assays

Microtiter 96-well tissue culture plates were coated with mouse laminin (Sigma Chemical Co.) at a concentration of 20 μg ml^{-1}, 0.1 ml per well, and incubated for 16 h at 4 °C. The laminin solution was aspirated and MEM with 1% BSA added, and incubated at 37 °C for 1 h. MDA-MB-435 (and other variants) cells were harvested by incubation with 0.02%-EDTA and single cell suspensions prepared in MEM with 1% BSA. Cells were incubated for 30 min with either rat IgG or α6 antibody (1:500), then 1×10^5 cells in 0.1 ml plated per well in the matrix-coated wells, or in untreated wells for the input values, and incubated for 3 h at 37 °C. Non-attached cells were removed by washing with

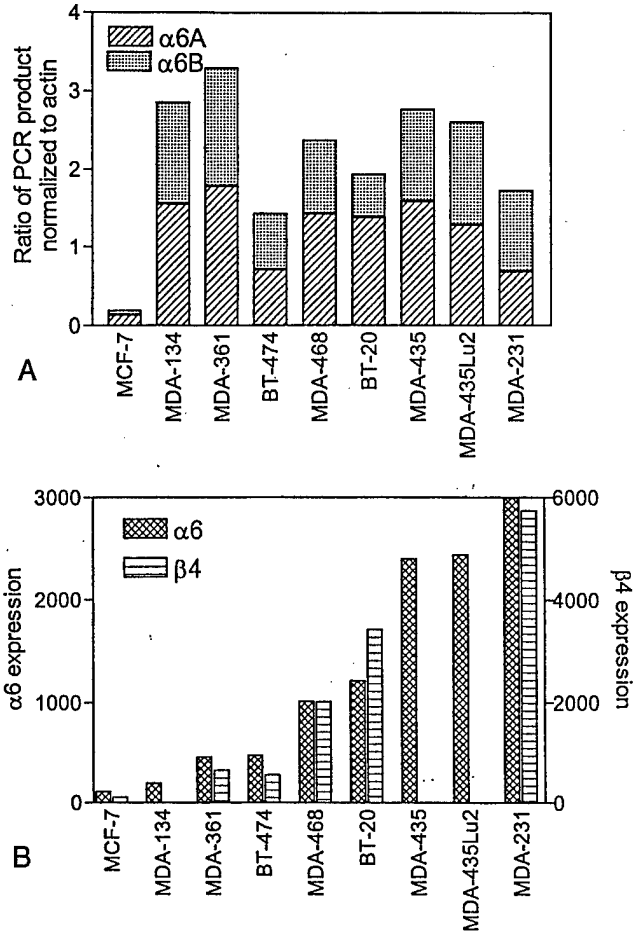

Figure 2. Relative abundance of α6 and β4 integrin sub-units on breast cancer cells. (a) Ratio of RT/PCR product of α6A and α6b normalized to β-actin (from Figure 1). (b) Protein expressed on the cell surface measured by flow cytometry, plotting the product of percent positive cells × MFU (from data shown in Table 1).

PBS, and the attached cell numbers quantified by incubation with MTT (Sigma Chemical Co.). The proportions of cells bound to the matrix-coated wells were calculated from:

$$\text{Percent bound} = \frac{\text{Absorbance from matrix bound wells}}{\text{Absorbance from input wells}} \times 100$$

Statistical analyses

The significance of differences in numbers of lung metastases was analyzed by the Mann–Whitney test. Differences in binding to laminin were analyzed using a two-sided unpaired Student's *t*-test.

Results

Expression of α6 integrin in breast cancer cell lines

The primers used in this study to detect α6 amplified two products, representing the α6A and α6B isoforms of the integrin. All of the cell lines expressed both isoforms, albeit at

Table 1. Expression of $\alpha 6$ and $\beta 4$ integrins by breast cancer cells as measured by flow cytometry.

Cell Line	$\alpha 6$		$\beta 4$	
	Percent positive[a]	MFU[b]	Percent positive	MFU
MDA-MB-231	98.8	30	99	58
MDA-MB-435	97.2	25	0	–
MDA-MB-468	90.8	11.2	98.9	20.4
BT-20	92.1	13.2	100	34.2
BT-474	59.5	7.92	95.5	5.8
MDA-MB-361	59.3	7.7	84.5	7.6
MDA-MB-134	44.9	4.3	0	–
MCF-7	27.3	4.1	18.7	6.1

[a]Percentage of cells expressing the antigen recognized by the monoclonal antibody to either $\alpha 6$ or $\beta 4$. [b]Mean fluorescence intensity expressed in relative fluorescence units. The results shown are representative of replicate experiments.

Table 2. Tumorigenicity and metastatic potential of breast cancer cells in nude mice.

Cell Line	ER expression[a]	Tumorigenic dose[b]	Metastasis[c]
MDA-MB-435	–	1×10^6	Yes (m.f.p.)
MDA-MB-231	–	2.5×10^6	Yes (i.v.)
MDA-MB-468	–	2.5×10^6	Infrequent
BT-20	–	5×10^6	No
BT-474	+	5×10^6	No
MDA-MB-361	+	5×10^6	No
MDA-MB-134	+	5×10^{6d}	No
MCF-7	+	5×10^{6d}	No

[a]ER expression from previously published reports [44, 46].
[b]Dose of breast cancer cells to consistently produce tumor growth in the mammary fatpad (m.f.p.) of female nude mice.
[c]Metastasis to the lungs or lymph nodes of nude mice following injection in the m.f.p. or i.v.
[d]Tumors grew progressively only when the mice also had s.c. implants of 17-β-estradiol.

different ratios; the $\alpha 6$A isoform is generally more abundant (Figures 1, 2a.). Measurement of the levels of $\alpha 6$ protein expression, by FACS analysis, also shows that all cell lines express this integrin sub-unit (Figure 2b, Table 1). The data from flow cytometry is expressed as the proportion of cells in the population that express the antigen, and also a relative measure of antigen density from the mean fluorescence intensity measured. The cell lines are listed in Table 1 in order of the level of expression, based on both percent positivity and the mean fluorescence intensity. A comparison of the levels of protein expression with the relative amounts of amplified products from PCR (normalized to the β-actin signal) show that there is concordance between the two assays for some cell lines (MCF-7, BT-474, MDA-MB-435) (Figure 2). For two cell lines however, (MDA-MB-134, MDA-MB-361) the protein expression is relatively low while RT-PCR analysis revealed relatively high levels of expression. This result suggested that $\alpha 6$ integrin expression in the different breast cancer cell lines may be regulated at the transcriptional and post-transcriptional level.

Expression of $\beta 1$ and $\beta 4$ integrins

The $\alpha 6$ integrin dimerizes with two β-subunits, $\beta 1$ and $\beta 4$. RT-PCR analysis revealed abundant expression of $\beta 1$ in all of the cell lines. With the exception of two cell lines, the expression of $\beta 4$ mRNA was readily detected. The exceptions were MDA-MB-134 cell line, which showed minimal expression, and the MDA-MB-435 cell line (and all variants derived from this line). In the MDA-MB-435 series of cell line a spliced variant was detected. Figure 3 shows that a 300 bp product was amplified, in addition to the 134 bp product amplified in all other cell lines, using the primers for nt 4679-4820. The variant PCR product was subcloned into TA vector and the DNA sequenced. Homology searching in the gene bank found no match for this alternatively spliced product. Amplification using the second set of primers resulted in the expected product in the MDA-MB-435 samples, confirming that the splicing event occured within the 4679–4820 nucleotide sequences.

Protein expression data for $\beta 4$ is shown in Table 1 and Figure 2b, and shows that the levels of this integrin sub-unit reflect the levels of expression of $\alpha 6$, with the exception of the two cell line that lack full length $\beta 4$ mRNA. No surface expression was detected in the MDA-MB-435 or MDA-MB-134 cell lines. Immunoprecipitation confirmed these findings. Precipitation with either $\alpha 6$ or $\beta 4$ antibodies revealed similar patterns of captured proteins, suggesting that in the majority of the breast cancer cell lines $\alpha 6$ dimerizes with $\beta 4$. In the MDA-MB-134 and MDA-MB-435 cells precipitation with $\alpha 6$ antibodies also precipitated $\beta 1$, showing that in the absence of $\beta 4$ the $\alpha 6$ subunit dimerized with $\beta 1$ (Figure 4).

Tumorigenic and metastatic properties of the human breast cancer cell lines

Previous reports from this laboratory have shown that the mammary fatpad of nude mice is an appropriate site for assessing the tumorigenicity of human breast cancer cells [24]. Table 2 summarizes the data from injecting breast cancer cells into nude mice, and shows the minimum dose of cells that are required to produce progressively growing tumors. The table also shows the metastatic potential of the cells, from either the mfp tumors (spontaneous metastasis) or from i.v. injection (experimental metastasis). With the exception of the HBL-100 cell line, that expresses high levels of $\alpha 6$ integrin (Figure 1, and data not shown) but is not tumorigenic in nude mice, our results show that the most aggressive cell lines (MDA-MB-435, MDA-MB-231) express the highest levels of $\alpha 6$. The MCF-7 cell line, which is used widely as a representative of an estrogen dependent breast cancer cell line, requires additional estrogen supplementation to support *in vivo* growth. Similarly, we have found that the MDA-MB-134 cell line will grow only in nude mice that have implants of 17β-estradiol. Even with the estrogen supplements, the minimum tumorigenic dose of cells required for progressive growth of these tumors (and others with lower $\alpha 6$ expression) is higher than the dose required for growth of the lines that have higher levels of $\alpha 6$ expression. The results show a

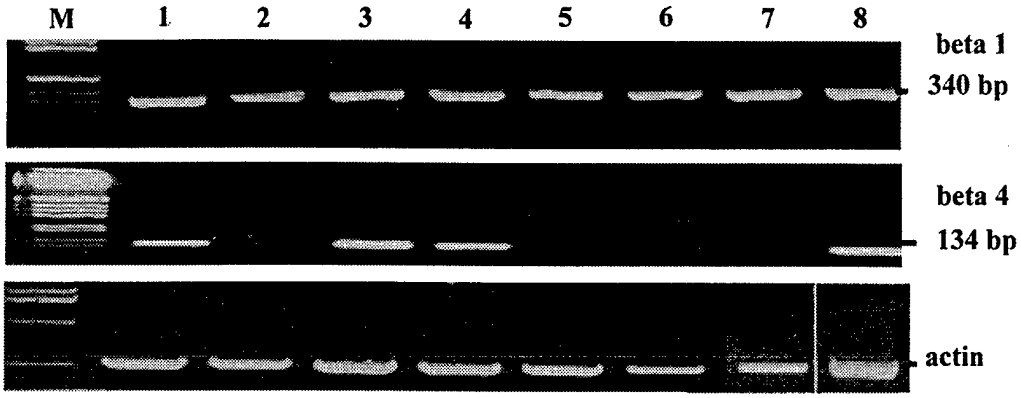

Figure 3. RT-PCR analysis of β1 and β4 integrins in human breast cancer cells. Legend: M, marker lane; lane 1, HBL-100; lane 2, MDA-MB-134; lane 3, MDA-MB-231; lane 4, MDA-MB-361; lane 5, MDA-MB-435; lane 6, MDA-435Lung2; lane 7, MDA-435Br1; lane 8, MDA-MB-468.

Table 3. Integrin expression and metastatic potential of MDA-MB-435 breast cancer variants.

Cell line	α6		α3		Metastasis		
	%[a]	MFU[b]	%	MFU	%[c]	Median#[d]	p value[e]
MDA-MB-435	97	25	90	2.5	60%	5 (0–20)	$p < 0.001$*
MDA-435Lung2	99	29	99	21.5	90%	20 (0–115)	
MDA-435Br1	68	2.4	100	26.5	10%	0 (0–8)	$p < 0.0001$*
MDA-α6LF9	75	4	81	2.9	36%	0 (0–55)	$p < 0.011$*
MDA-α6HG6	99	33	99	26.7	75%	10 (0 > 150)	$p = 1.0$

[a]Percentage of cells expressing the antigen recognized by monoclonal antibodies to either α6 or α3 integrin subunits.
[b]Mean fluorescence intensity expressed in relative fluorescence units.
[c]Number of mice with metastasis/number of mice with mammary fatpad tumors × 100.
[d]Median number (and range) of macroscopic lung metastases.
[e]Results of Mann Whitney tests comparing the numbers of metastases with those in the mice with MDA-435Lung 2 tumors. The variants with higher α6 expression produced significantly more lung metastases.

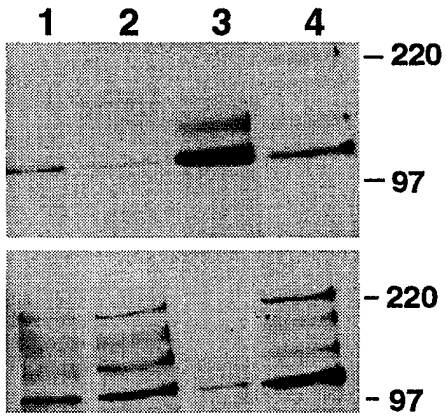

Figure 4. Immunoprecipitation of α6 and β4 integrins. Lysates of biotin-labeled cells were subjected to immunoprecipitation with anti-α6 (upper panel) or anti-β4 (lower panel) specific antibodies. Legend: lane 1, MDA-MB-361; lane 2 MDA-MB-468; lane 3, MDA-MB-435, lane 4, MDA-MB-231.

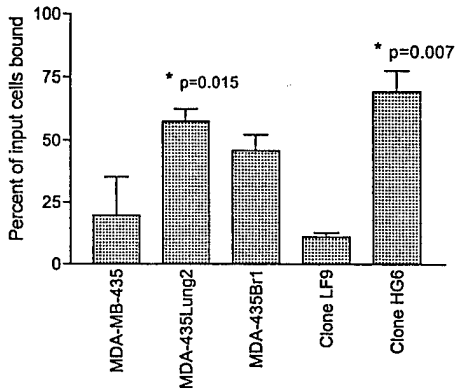

Figure 5. In vitro binding of MDA-MB-435 cells to laminin. The percentages of the input number of cells attached after 3 h incubation on laminin-coated surfaces are shown. Significantly more MDA-435Lung2 and MDA-α6HG6 cells bound to laminin compared with the original MDA-MB-435 cell line (Student's *t*-test)

relationship between α6 expression and malignant potential of human breast cancer cells.

Metastatic potential of selected clones and variants of MDA-MB-435

The MDA-MB-435 cell line is highly metastatic in nude mice, has high levels of α6, yet lacks β4 expression. Variants of the cell line have been isolated from metastases in nude mice, and one isolated from a brain metastasis (MDA-435Br1) was found to have reduced metastatic properties [23]. When the α6 levels were measured on this variant, lower expression was found compared with the original line, and with a variant isolated from a lung metastasis (Table 3). Clones were isolated on the basis of high or low expression of α6, by sterile sorting of cells incubated

with monoclonal antibody, and differences in levels of expression of the integrin were confirmed. The MDA-α6HG6 clone and MDA-435Lung 2 variant (high expression of α6) showed significantly higher binding to laminin (Figure 5) than the original cell line and the MDA-α6LF9 (low-α6-clone). Binding to laminin was abrogated by the addition of the antibody to α6 (data not shown). The variants expressing highest levels of α6 integrin produced significantly more lung metastases in nude mice than the low-α6 expressing cells (Table 3), although the growth rates of the different variants in the mfp did not differ (data not shown). Thus, the spontaneous metastatic potential of the MDA-MB-435 breast cancer cell line is associated with high levels of α6 expression. High α6 expression also correlated with binding affinity to laminin *in vitro*, with the exception of the MDA-435Br1 cell line, which has lower α6 expression yet binds well to laminin (Figure 5). One explanation for this inconsistent finding is that the MDA-435Br1 cells express high levels of α3, which forms a dimer with β1 and mediates binding to laminin. Within the series of MDA-MB-435 variants, α3 expression can mediate laminin binding (antibody to α3 inhibited binding, data not shown) but the level of expression was not correlated with metastatic potential (Table 3).

Discussion

Altered integrin expression has been linked to the malignant phenotype of a variety of cancer types [4, 5]. These observations have been interpreted as further evidence of disordered communication between malignant cells and their immediate microenvironment. Elevated expression of α6 integrins has been associated with malignant transformation of mouse skin [25] and the invasive and metastatic phenotypes of various rodent and human cell lines [9, 10, 26–28]. This study focused on the expression of α6 integrin and associated β sub-units in established breast cancer cell lines. Our experimental results reflect the clinical findings [18, 19] with the highest expression of α6 in the most aggressive cell lines, tested in a nude mouse model of breast cancer growth and metastasis.

As the different cell lines differ in many other phenotypes that might affect malignant growth potential in nude mice, we compared the α6 expression and metastatic potential of isogenic variants derived from the MDA-MB-435 breast cancer cell line. The cell line is heterogeneous for expression level of the α6 integrin, and also for metastatic phenotype. Selection of cells from lung metastases in nude mice resulted in a cell line with high metastatic ability to the lungs of nude mice, and with high α expression. Further, a clone selected for high expression of the α6 integrin had high metastatic potential when injected into nude mice. Conversely, the variant and clone with low α6 integrin expression produced significantly fewer lung metastases. Tumor growth in the mammary fatpad, and invasion of cells through matrigel coated filters [29] were not different for these variants, suggesting that the elevated expression of the α6 gave the breast cancer cells an advantage at later stages in the metastatic cascade, i.e. the arrest, extravasation and growth in the new organ (in this instance the lungs). In a previous study the high α6-expressing MDA-435Lung2 cells showed greater binding affinity to lung endothelial cells than the low α6-expressing MDA-435Br1 cells [29]. Another study using the MDA-MB-435 cell line reported that α6 expression provided a survival advantage for the metastatic cells in nude mice [30]. Our results concur with this previous result that α6 expression on the breast cancer cells is associated with enhanced metastatic potential.

The α6 sub-unit is preferentially expressed as a dimer with β4 in the breast cancer cell lines. β4 is known to dimerize only with α6, thus as might be expected the levels of the two integrin sub-units were of similar order of rank in the cell lines tested. Two of the panel of cell lines lacked β4 protein expression, and in these cells α6 dimerized with β1, to form the VLA-6 laminin binding receptor. The reason for the loss of β4 expression in MDA-MB-134 cell lines is currently unknown. There are previous reports of alternative splicing of the cytoplasmic tail of β4 in normal and malignant epithelial cells [20, 22], that were compatible with protein expression. For the MDA-MB-435 breast cancer cell line we report a novel variant of the cytoplasmic region of β4. While low levels of the normal transcript were detected in RT-PCR reactions of cDNA from MDA-MB-435 variants, the presence of the alternately spliced form presumably eliminated translation of the β4 protein, or reduced it to below the level of detection.

β4 is an unusual integrin sub-unit in that it has a long cytoplasmic domain, ~1000 aa, compared with the ~50 aa in other β subunits [22]. Binding sites for adapter proteins and tyrosine phosphorylation sites have been identified in the β4 cytoplasmic domain, suggesting unique signalling functions for this integrin [31]. Fibronectin like domains in the cytoplasmic tail are necessary for association with elements that form the hemidesmosomes [32], which are expressed on the basal aspect of normal mammary epithelial cells. There are conflicting reports concerning the role of the β4 sub-unit in malignancy. α6β4 mediates binding to laminin-5, which is found expressed at invading fronts of some carcinomas [33]. The introduction of a normal β4 into a human colon cancer line and the MDA-MB-435 breast cancer cells enhanced their invasion potential through Matrigel-coated filters [34]. Conversely, the transfection of β4 into another colon cancer cell line resulted in induction of $p21^{WAF1/CIP1}$ and growth arrest [35], and complete suppression of growth of a human bladder carcinoma [36]. In our study the level of β4, paralleling the level of α6, was highest in the more tumorigenic breast cancer cell lines, which argues against a tumor-suppressive role for β4 in the breast cancer cells. However, the results with the MDA-MB-435 line clearly show that β4 expression is not required for invasion and metastasis. One possible explanation for the apparently conflicting results in the different studies may be the potential for alternate splicing that can lead to variant forms of the cytoplasmic tail of β4 [22, 37, 38]. These variants may then differ in the capacity for associating with adapter proteins

or other intra-cellular elements, and thus lead to different phenotypes.

The mechanisms regulating α6 expression in different cell types are currently unknown. TGF-β1 is reported to increase α6 expression, while TNF-α and IFN-γ can depress expression in various cell types [39–41]. The inverse relationship we show between ER expression and α6 may suggest that the integrin levels may be, at least in part, hormonally regulated. The promoter region of the human α6 gene has been cloned and reported by two independent groups and both report the presence of elements that are responsive to steroids [42, 43]. One inference from our observation is that α6 expression may be regulated by means of steroid receptors, directly or indirectly. On going studies are testing this possibility.

An alternative explanation for our observation is that the ER-negative, high α6-expressing cells represent cells that have undergone EMT [44]. The patterns of expression seen in the breast cancer cells may be the result of co-ordinate regulation of ER and α6, and not that integrin expression is modified by signalling through ER. Alterations in other adhesion molecules, such as E-cadherin, have been presented as examples of the shift in phenotype associated with EMT, which may be a critical step in the malignant progression of breast epithelium [45]. The increased levels of α6 may be an indicator of EMT by the breast cancer cells, as only one of a variety of phenotypic changes, rather than contributing directly to malignant progression. Other studies have provided evidence of a role for this integrin by showing that direct modification of α6 expression or function can alter invasion and metastasis [9, 10, 30], although we recognize that this is only one of many molecules involved in the metastatic process. It remains to be established whether the level of α6 expression in breast cancer cells is a reflection of EMT, or is regulated by steroids or other factors in the tissue microenvironment. However, the data presented show a clear association between expression of this integrin sub-unit and the malignant potential of breast cancer cells. This reproduces the clinical situation of a poor prognosis for women with breast cancers that express high levels of α6 [18, 19]. This model of human breast cancer metastasis can be used for futher analyses of how α6 integrins mediate metastasis and for the development of novel intervention strategies for treating or preventing disseminated disease.

Acknowledgements

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Differential effects of osteopontin antisense RNA on the growth of human breast cancer cells in vitro and in vivo[1]

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Abstract

Osteopontin (OPN) is a secreted phosphoprotein expressed by many normal and transformed cells. Variants of the MDA-MB-231 human breast cancer cell line derived from metastases in nude mice expressed higher levels of OPN mRNA than the original cells. An antisense OPN construct was made into pRc/CMV and transfected into lung-metastasis derived MDA-231-LC1 cells. Stable clones were generated after neomycin selection. Antisense RNA inhibited 50 – 60% of endogenous OPN RNA and protein, as measured by RT-PCR and western blot analyses. In cell proliferation assays, the antisense-transfectants showed 50 – 60% reduced growth rates in colony formation assays, and were more sensitivity to etoposide and staurosporine. In addition, treatment of these cells with an antibody that recognizes the $\alpha v\beta 3$ integrin partially inhibited growth, implying that interaction between OPN and this integrin may regulate growth and survival of these cells. The clones with reduced OPN expression formed tumors in the mammary fatpad of nude mice at a similar frequency as the control cells, yet the potential for experimental metastasis to the lungs of nude mice was increased. Our results suggest the function of OPN in malignant progression of breast cancer cells is complex and may depend upon the growth environment.

Introduction:

Interactions between cancer cells and the extracellular matrix can have important regulatory actions on malignant growth and dissemination. A variety of cell surface receptors and ligands present in the extracellular environment can initiate intra-cellular signals, and effect changes in gene expression and cellular phenotype. Osteopontin (OPN) is a secreted glycoprotein, expressed by normal cells but also known as a marker of transformed cells with elevated expression in malignant cells and tissues (1-3). OPN contains the cell attachment motif arginine-glycine-aspartic acid (RGD), and is a known ligand for cell surface integrins, including the αvβ3 heterodimer(4-7). As a ligand for multiple receptors, OPN is thought to influence signaling pathways and cellular processes. For example, OPN may promote bone resportion as it mediates binding of osteoclasts through the αvβ3 integrin and can stimulate Ca2+ efflux (4). OPN has also been shown to alter the phosphorylation status of cytoskeletal proteins and focal adhesion kinase, and to induce the activity of pp60 src kinase in human melanoma cells (5; 8). OPN levels were shown to be elevated in the plasma of women with metastatic breast cancer, and increasing levels of the protein were associated with shorter patient survival (9). How elevated OPN expression may affect breast cancer growth is not known. Variants of the human breast cancer cell line MDA-MB-231 derived from metastases in nude mice were found to express elevated levels of OPN mRNA (Price *et al*, in preparation). To test role of OPN in the malignancy of breast cancer cells, a plasmid vector was constructed to express antisense mRNA, to determine how manipulating the OPN level would affect the growth of the breast cancer cells.

Methods

Cell culture: The breast cancer cells were cultured in Eagle's minimum essential medium supplemented with 5% FCS, non-essential amino acids, L-glutamine, sodium pyruvate and MEM-vitamins (GIBCO-BRL, Grand Island, NY) and maintained in monolayer culture in a humidified 37°C incubator with 5% CO_2-95% air atmosphere. The MDA-231 LC1 (from lung metastases in a nude mouse injected i.v. with MDA-MB-231 cells) expressed an increased level of OPN compared with the original cell line, and was used for the transfection studies.

Construction of antisense Osteopontin in expression vector:The 1.2 kb of human osteopontin coding region was subcloned from bluescript (obtained from ATCC, Rockville, MD) and ligated into the mammalian expression vector (Invitrogen, Carlsbad, CA). Subcloning was done in two steps. The full length DNA fragment was recovered from polymerase chain reaction by using Hind III linker at both 5′ and 3′ end. It was then ligated into TA vector (Invitrogen). The Hind III digested 1.2 kb fragment was rescued and placed into pRc/CMV expression vector. Restriction digestion and sequencing confirmed the antisense orientation of the insert in a construct designated CMV-AS OPN.

Establishment of antisense OPN expressing cells: MDA-231 LC1 ($1\text{-}5x10^5$) cells were transfected with 1-2μg of recombinant plasmid DNA using Fugene, following the manufacturer's recommended procedure (Boehringer Mannheim, Indianapolis, IN). The pRc/CMV expression vector contains a neomycin selectable marker to select G-418 resistant clones. Forty-eight h after transfection, 1mg/ml G-418 was added and individual G-418 resistant colonies were isolated and expanded for further studies.

Extraction of genomic DNA: High molecular weight genomic DNA was isolated using a standard technique (10). Cells were lysed in STE buffer (0.1M NACL,10mM Tris HCl,pH 8.0,

1mM EDTA) containing 1% SDS and 200μg of proteinase K/ml at 37°C overnight. DNA was then extracted with phenol/chloroform and precipitated with ethanol, washed with 70% ethanol, air dried and dissolved in TE buffer.

RNA extraction and Northern blot analysis: Total RNA was extracted from cells using Trizol reagent (Sigma Chem.Corp., St. Louis, MO) Total RNA (20 μg) was size fractionated in a 1.2% agarose gel using MOPS/formaldehyde buffer. RNA was transferred onto a GeneScreen membrane and was hybridized with a full length OPN DNA probe that was α-^{32}P-labelled (dCTP, 3000 Ci/mmole; ICN Biochemical Inc., Irvine,CA) by random priming (Ambion Inc., Austin TX)

RT/PCR and southern blot analysis: cDNA was synthesized from 5 μg of total RNA using oligo dT as primer. cDNA or 100 ng of genomic DNA was used to amplify exogenous OPN DNA using 1 μM each of the 5′ T7 primer of the pRc/CMV vector and the 3′ OPN primer, 200 μM of each dNTPS and Taq 1 DNA polymerase (1u/100ul). We have used β1 (11) and β3 integrin oligos (12) to test the downstream effect of endogenous antisense OPN expression in these clones. In some instances 5′ and 3′ primers corresponding to the OPN DNA were used to amplify endogenous cDNA by PCR. Amplified PCR products were size fractionated on 2% agarose gel by electrophoresis, stained with ethidium bromide, visualized and photographed under UV transilluminator. High molecular weight genomic DNA (30μg) from control and antisense transfected clones were digested with Hind III. The DNA fragments were size fractionated onto GeneScreen membrane (Amersham, Arlington Heights, IL) and hybridized with radioactive labeled 1.4 kb Xba I and Pst I OPN DNA probe.

Cell proliferation and clonogenic assays: Using 24 well culture plates, $2x10^4$ cells were seeded per well in MEM supplemented with 5% FCS. At 24-h intervals over five days, cells

were trypsinized and counted using a hemocytometer. All experiments were done in triplicate, and results expressed as mean ±S.E. of 3 replicates. For clonogenic growth assays, $3x10^3$ exponentially growing cells of control and antisense OPN transfectants were suspended in 1 ml of 0.35% Seaplaque agarose in MEM medium with 10% FCS and plated on top of a 1ml underlayer of 0.75% agarose in the same medium in a 60 mm-tissue culture plate. Cell colonies of greater than 50 u diameter were counted after 2 weeks of incubation. In the monolayer colony formation assay, 500 cells were plated in MEM without G-418 medium in 60 mm plates. After 3 weeks of incubation the colonies were stained with Geimsa and colonies were counted visually, and the mean ± S.E. of 3 replicate plates calculated.

Western blot analysis: Control and antisense transfected cells were harvested and placed in 1x SDS PAGE loading buffer and boiled for 5 minute. A total 30 μg protein/lane was then electrophoresed through a 12% denaturating polyacrylamide gel and electroblotted onto a membrane. OPN protein was detected immunochemically using the OP-189 antibody generously provided by Dr. Cecilia Giachelli (3). The blots were simultaneously probed with monoclonal antibody actin as a loading control.

Flow Cytometry: Cells were harvested with trypsin/EDTA , washed once with MEM containing 10% FCS and incubated in PBS containing saturating concentrations of αvβ3 antibody (LM 609, from Chemicon, Temecula, CA) and αv (Upstate Biotechnology Incorporated, Lake Placid, NY) for 30 min. at 4^0 C. After washing twice with PBS , cells were incubated with FITC-conjugated goat anti-mouse antibody (Sigma Chemical Co.) as appropriate for 30 min.at 4^0 C. Cells were washed twice with PBS and subjected to flow cytometry using a EPICS ELITE analyzer.

Blocking experiment with antibody to αvβ3: To study the relationship of OPN and αvβ3 integrin on clonogenic potential at low seeding density, OPN antisense transfectants and control cells were plated in 96-well plates at a density of 500 cells/well with MEM and 5% FCS medium. Cells were treated with 10 μg/ml mouse IgG or 10μg/ml αvβ3 antibody (LM 609). Cell growth was monitored by MTT assay. The values are the mean ±S.E. of four replicates. Inhibition in the presence of αvβ3 antibody was expressed as a percentage of absorbance of the normal IgG treated cells.

Apoptosis analysis and Chemosensivity assay: Cells ($1x10^6$) were seeded and incubated for 6 h, in suspension in microcentrifuge tubes or plated in tissue culture plates. Etoposide (80μm, Sigma) was added for 48 h, or staurosporine (20 nm, Sigma) for 18 h. Apoptotic cells were detected using the ApopNexin kit, following the manufacturer's recommended procedure (Oncor, Inc., Gaithersburg, MD). Cells stained with the FITC-annexin and propidium iodide solutions were analyzed in a flow cytometer; early and late apoptotic cells were counted as dead cells. Trypan blue dye exclusion was also used to determine the viabilty of the cells attached or in suspension (attached cells were harvested by trypsin-EDTA treatment), counting cell suspension in a hemocytometer chamber.

RESULTS:

Isolation of human breast cancer cell clones stably transfected with antisense OPN expression vector:

MDA-231 LC1 cells were transfected with the antisense OPN recombinant plasmid construct and transfectants were selected for neomycin resistance. Genomic DNA was isolated from the transfected clones and the presence of exogenous OPN DNA was determined by PCR

analysis as shown in Fig. 1a. We used a 5′ oligonucleotide primer corresponding to the T7 sequence of the pRc/CMV vector and a 3′ primer from the OPN DNA sequence in order to detect only the introduced vector sequence. A 400 bp DNA fragment, the predicted size of specific product was amplified from the clone derived from transfection with CMV- OPN plasmid DNA. We determined expression in three cell lines shown in the figure. Neomycin-resistant clones of cells transfected with pRc/CMV vector only did not yield a PCR product. In Fig.1b. southern blot hybridization showed genomic integration of plasmid DNA . AS-OPN CL#3 has more than one copy of plasmid DNA integrated into the genome.

Expression of endogenous OPN mRNA: Total RNA was extracted from three clones derived from 231-LC1, AS-OPN-CL#1, -CL#3 and -CL#5 and control cells to analyze the expression of OPN mRNA. An aliquot of cDNA was used to amplify OPN sequences using the appropriate oligonucleotide PCR primers. A 400-bp product was amplified using the internal primers that were designed to detect total OPN transcripts. Lower levels of OPN transcripts were detected in AS CL#3 and AS CL#5 compared to vector control in Fig.2a. The β-actin control showed that comparable amounts of RNA were present in each sample. The expression of OPN mRNA in the antisense transfected clones was examined by northern blot analysis to establish the approximate size of the OPN transcript. The OPN transcript was detected just above the position of 18S ribosomal RNA. No truncated species were observed in the transfected clones (data not shown).

We assessed the downstream effect of altered OPN expression by assessing the expression of the integrins β1 and β3 by RT/PCR analysis. Several OPN receptors, including αVβ1 and αVβ3 have been associated with tumor progression. Expression of β3 mRNA was significantly reduced in antisense clones when compared to control, whereas β1 mRNA expression remained unchanged as shown in Fig.2b.

Down regulation of endogenous OPN protein in antisense transfected cells : To measure the expression of OPN protein in the antisense-transfectants, cells were lysed and OPN protein was detected (68 kd) by western blot analysis (Fig. 3). We detected an approximtely 2.5-fold decrease in the level of OPN expression in those antisense clones as compared to control. As OPN is known to be a secreted phosphoprotein, we also detected protein in conditioned media, with volumes normalized with equal number of cells separated on SDS-PAGE gels (data not shown). The results showed that there were reduced levels of OPN protein in the antisense transfected clones compared to vector control clones.

In vitro cell growth: Cell growth kinetics on tissue culture dishes were compared between neo control and antisense transfected cells. Vector control showed longer initial log phase than antisense clone. There is a significant different between control and antisense transfectants in the growth at mid-log phase (data not shown). The properties of cells remain unchanged after more than 12 passages. The ability to form colonies in soft agarose is a reflection of malignant transformation (13). Therefore, to assess the effect of OPN expression on the malignant properties of MDA-231 LC1 cells, we compared the ability of control (Vector#3) and antisense-transfected clones to form colonies in soft agarose as shown in Fig.4a. All antisense clones had a significantly lower cloning efficiency (50-60%) in semisolid medium than control. In addition, we compared the ability of the control and OPN antisense-transfected cells to form colonies at low seeding density. In Fig.4b, AS-clone #3 showed a significant reduction of cloning efficiency (50-60% of the neo control respectively). This suggested that antisense OPN can inhibit cell proliferation under stringent growth conditions of clonal growth or in semi-solid agarose.

Interaction of αvβ3 and osteopontin : We tested possible interactions between αvβ3 and OPN in living cells using the MTT assay and flow cytometry. 500 cells were plated and incubated with or without αvβ3 for one week and compared with control. Growth of antisense CL#3 cells was reduced by 50%, when compared to vector control (Table 1). The antisense clones express less OPN and αvβ3 (measured by flow cytometry) but are more sensitive to the inhibitory effects of the αVβ3 antibody. One possible explanation is that a reduced level of OPN allows more antibody to bind to the receptor, whereas excess OPN reduces or competes for binding to the integrin. Flow cytometry of cells incubated with a primary antibody to the αv integrin, rather than the αvβ3 heterodimer, did not reveal any differences in expression. The αv sub-unit can dimerize with several different β-subunits. Apparently the reduction of αvβ3 expression in the antisense OPN clones may be a function of suppressed β3 expression (see above), and not on total levels of αv integrin

Apoptosis sensitivity: A variety of signals can trigger programmed cell death, including altered interactions with the environment, possibly mediated through cell-surface receptors and the extracellular matrix (14). To test whther the reduced clonogenic growth potential of the antisense-OPN clones was a reflection of altered sensitivity to apoptosis inducers, the cells were exposed to etoposide or staurosporine. Etoposide is thought to act via topoisomerase,and staurosporine is a protein kinase blocker that induces apoptosis in a variety of mammalian cell types (15). Cell viability was measured by Trypan blue dye exclusion of cells incubated in suspension with the drug, or cells were collected and stained with FITC-labeled annexin V and propidium iodide, to measure numbers of apoptotic cells. Etoposide treatment (80 μm, for 48 h) reduced the viability of AS Cl#3 cells by 55%, with no affect on the control cells at this concentration (higher drug concentrations we equally toxic to both cell lines). The number of

apoptotic AS Cl#3 cells increased four-fold with etoposide treatment, with only a two-fold increase seen in the control transfected cells. Staurosporine treatment (20 nm for 18 h) produced a 60% drop of viability of the AS Cl#3 cells in suspension, with 25% reduction in the control transfected cells. However, there were no differences in the numbers of apoptotic cells following staurosporine treatment, attached or in suspension. For both agents, the cells with reduced OPN were more sensitive to killing when incubated in suspension (an anoikis assay), compared with growth attached to tissue culture plastic.

Tumorigenicity and experimental metastatic potential: The mammary fatpad of nude mice has been shown previously to be a favorable site for the growth of human breast cancer cells(16). Table 2 shows the incidence of tumors of the antisense-OPN clones, with only 10 or 20% incidence of growth from injection of the transfected cells. This was a lower tumor take rate than found with non-transfected MDA-231 LC1 cells (data not shown). However, the experimental metastasis assays showed that the antisense OPN-breast cancer cells had significantly greater ability to form colonies in the lungs of nude mice (Table 2). Both *in vivo* assays were performed three times, giving similar results. Analyses of genomic DNA samples from AS Cl#3 and AS Cl#5 lung tumors showed that the cells retained the introduced antisense construct (PCR and Southern blot analyses performed as described above). Thus, altered levels of OPN had different effect on the growth of the MDA-231 LC1 cancer cells, depending on whether the cells are growing *in vitro* or *in vivo.*

Discussion

Elevated OPN expression has been associated with malignant transformation of many cell types, although the function of the protein in promoting tumor growth and metastasis has not been fully defined. Functional studies have been limited, for the most part, to established

transformed cell lines. Previous reports using sense or antisense constructs to modulate expression have generally supported the idea that OPN expression promotes tumor cell growth, *in vitro* and *in vivo* (5; 17-19). In contrast, a recent study using OPN-null mice exposed to a carcinogen suggested that tumor growth and metastasis was more rapid and more frequent in the absence of OPN. Tumor cells derived from OPN-null mice retained high tumorigenic potential when transplanted into nude mice, and showed reduced clonogenic growth when OPN was introduced into these cells(20). The authors concluded that the function of OPN in malignant progression is more complex than previously thought, and may be due to different functions of this protein in normal and malignant cells. The protein is reported to undergo post-translational modification, possibly resulting in protein forms with different functions, or that bind different receptors on various cell types, and generating different signaling pathways (21-24).

In this report we altered OPN expression in human breast cancer cells using an antisense construct with a heterologous (CMV) promoter. The reduced expression of OPN RNA and protein corresponded with reduced clonogenic growth in vitro, and increased sensitivity to inhibition by antibody to $\alpha v\beta 3$ and cytotoxic agents. However, the metastatic capability of the cells with reduced OPN was increased, compared with the control transfected cells, which is in contrast to what has been described with other cell systems, and what might be expected from the *in vitro* findings.

OPN has been shown to enhance the survival of endothelial cells in vitro (25) and may contribute to endothelial cell growth and thus angiogenesis at sites of tumor growth. OPN is also a known chemoattractant for macrophages, and immunohistochemical studies have localized expression to the leading edge of invasive human cancers, in both malignant cells and macrophages (26). It has also been shown that OPN can protect tumor cells from macrophage

mediated killing *in vitro*, by inhibiting nitric oxide production (27; 28). Macrophages detected in mouse OPN-producing tumors were thought to be unactivated, based on the expression of mannose receptors (20). Thus while OPN may promote the presence of macrophages in the tumor tissue, it may protect the tumor cells from cytolytic action of these host cells. Infiltrating lymphoid cells and macrophages can produce growth and angiogenic factors, and hence contribute to angiogenesis (29; 30). Mouse macrophages can be detected in human tumors growing in nude mice (31; 32). Whether modifying OPN expression in MDA 231-LC1 breast cancer cells resulted in differences in macrophage infiltration of the tumors in the mammary fatpad or the lungs, which might affect the ability of the cells to grow in these tissues, is currently under investigation.

The reduced clonogenic growth of the OPN antisense-transfected breast cancer cells may suggest an autocrine function of this protein in promoting growth and/or survival. Initial data from RT-PCR analyses showed that OPN may regulate the β3 integrin expression in these cells, a finding seen in endothelial cells (33). In the cells with reduced OPN expression, and with lower αvβ3 surface expression, a blocking antibody to the integrin reduced clonogenic growth. The same antibody blocked binding of MDA-MB-231 breast cancer cells to immobilized OPN (Price *et al.*, in preparation), suggesting that OPN can compete with the antibody for binding to the integrin heterodimer. The antisense clones, with lower OPN expression, may thus be more sensitive to the inhibitory effects of the integrin blocking antibody. Petitclerc *et al.* (34) suggested that melanoma cell survival regulation through the αvβ3 integrin is mediated by altered bcl-2:bax protein ratios. Whether OPN mediates changes in the levels of the bcl-2 related family of apoptosis mediators in the breast cancer cells is currently unknown.

In conclusion, we found that reduction of OPN from introduction of an antisense construct into a breast cancer cell line corresponded with reduced clonogenic growth, and increased sensitivity to growth inhibitory effects of a blocking antibody to the αvβ3 integrin. However, the cells with reduced OPN formed significantly more lung metastases in nude mice following i.v. injection. While OPN has been associated with malignant transformation and progression, the function of this protein in regulating growth of human breast cancer cells is still unclear. The results of this study indicate that how OPN may influence cell growth or survival may depend upon the environment.

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Table 1: Expression of αvβ3 integrin by MDA-231 LC1 cells and sensitivity to growth inhibition by antibody

Cell Line	Percent positive[a]	Growth in presence of IgG[b]		
		Control (IgG)	αvβ3	% inhibition
Vector #3	14.9	0.98 ± 0.1	0.78 ± 0.04	20
AS Cl#1	11.8	0.56 ± 0.04	0.33 ± 0.07	41
AS Cl#3	3.5	0.44 ± 0.1	0.2 ± 0.03	54
AS Cl#5	9.1	0.42 ± 0.05	0.4 ± 0.04	5

a Percentage of cells expressing the αvβ3 antigen, as recognized by the LM609 antibody (1:50 dilution). Flow cytometry was performed on an EPICS Profile Cell Sorter with a 525-nm band pass filter to detect FITC and gated on forward *versus* side scatter to exclude debris, dead cells and clumps. Analysis was based upon cursors set at 2% for background staining with isotype matched control IgG.

b: Absorbance values from MTT assays of cells plated with control IgG or LM609 antibody. Mean and S.D. are shown for three replicate plates, and are representative of three experiments.

Table 2: Tumorigenicity and metastatic potential of MDA-231 LCI antisense clones

Cell line	Incidence of mfp tumors[a]	Incidence of lung metastases[b]	Median# (range)[c]
Vector #3	5/14	13/24	6 (0 – 230)
AS#3	3/15	19/23	80 (0 – 250) p = 0.026
AS#5	1/10	13/13	105 (2 – 250) p = 0.013

a: Number of mice with tumors/number of mice injected with 2.5 x 10^6 cells into the mammary fatpad of female nude mice.

b: Number of mice with experimental lung metastases/number of mice injected with 1 x 106 cells into the lateral tail vein of female nude mice. Mice were killed 8 weeks later (or when moribund) and the numbers of macroscopic lesions in the lungs were counted.

c: Median number and range of experimental lung metastases. The p values shown are from Mann-Whitney tests comparing the numbers of metastases with those in the lungs of mice injected with the MDA-231 LC1-vector#3 cells.

Figure Legends:

Figure 1 a: DNA-PCR analysis of antisense OPN DNA integration into the stable transfectants

Figure 1b: Southern blot analysis of OPN in stable transfected clones

Figure 2a: RT-PCR analysis of OPN transcripts in the vector (V#3) and antisense (AS) clones, using primer for OPN (as described in methods and materials) and for β-actin as reference. The bar graph shows levels of OPN transcripts normalized to actin transcrpit expression, from drensitometric analyses.

Figure 2b: RT-PCR analyses of β1 and β3 integrin expression in the antisense transfected cells. Amplification mixtures contained primers for the integrin and for GAPDH as a reference.

Figure 3: Expression of OPN protein in MDA-231 LC1 antisense clones by immunoblot of cell lysates. The proteins were identified by hybridization with OP-189 antibody. The same filter was hybridized with antibody recognizing actin, to show equal loading. The bar graph shows the levels of OPN protein normalized to the actin, from densitometric analyses of the immunoblots.

Figure 4: a)Agarose colony growth of the MDA-231 LC1 antisense clones. The numbers of colonies of >50μ diameter were counted after 21 days incubation. b)Clonogenic growth resulting from plating 500 cells per 60-mm tissue culture plate. After 21 days incubation, the plates were stained with Geimsa, and colonies counted. Results shown are mean and S.D. (bar) of triplicate wells, and are representative of three separate experiments.

Figure 1a: DNA-PCR analysis of antisense OPN DNA integration into the stable transfectants. (M = molecular weight marker; V= vector control clone; AS1,3,5 = antisense clones #1,3 and 5; +Con = positive control plasmid DNA)

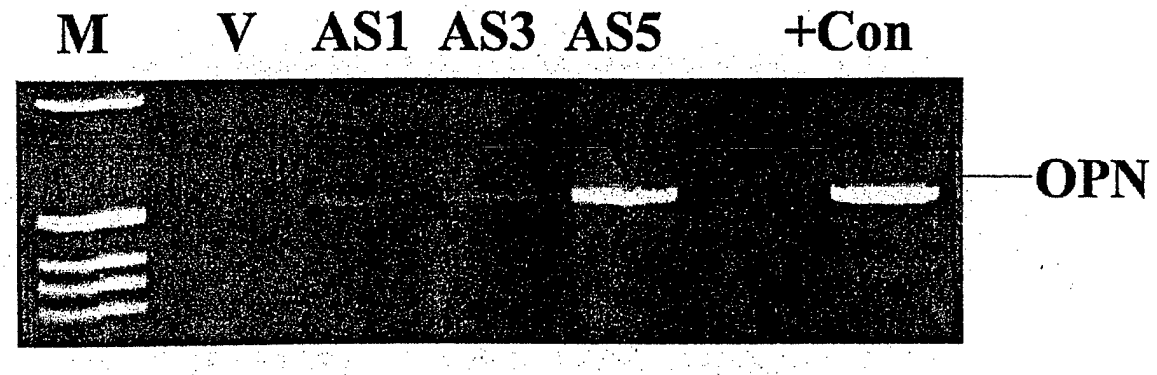


Fig. 1b: Southern blot analysis of OPN in stable transfected clones

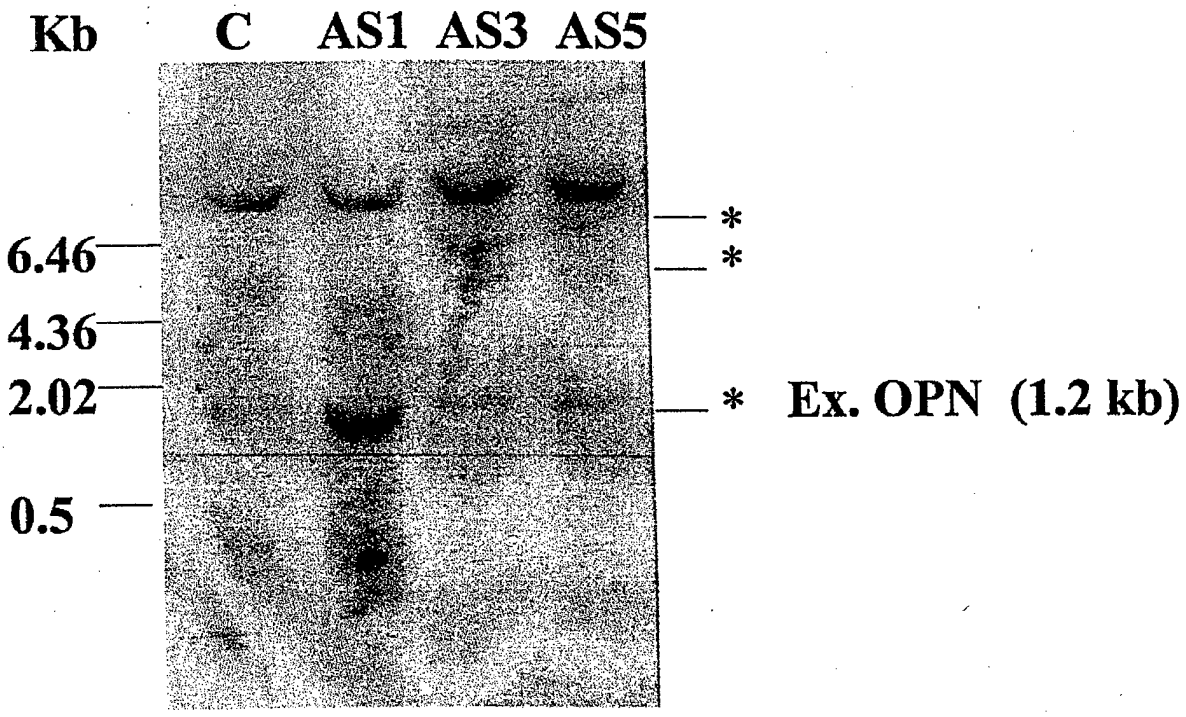


Fig.2a

OPN (400 bp)

Actin

V#3 AS#1 AS#3 AS#5

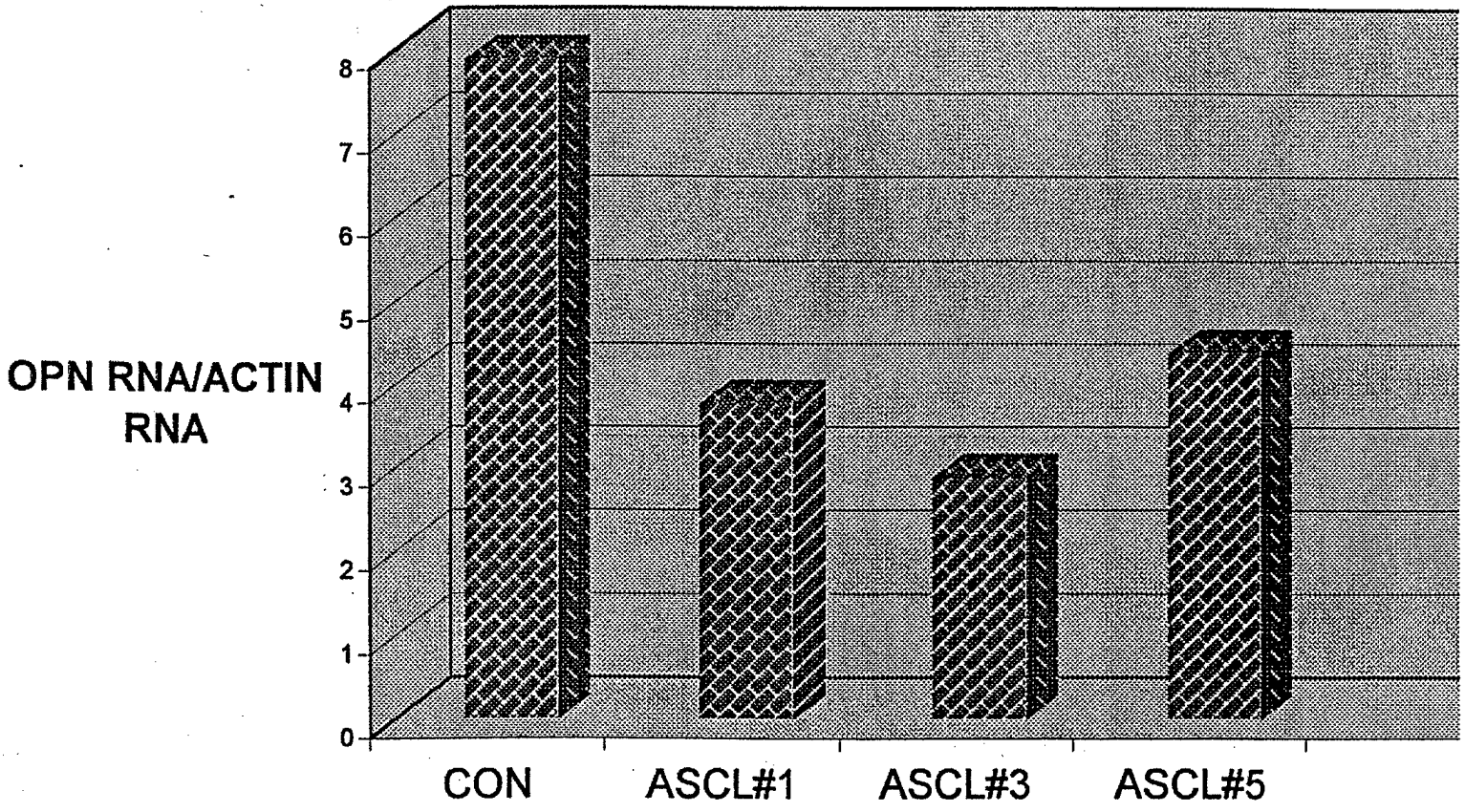


Fig. 2b

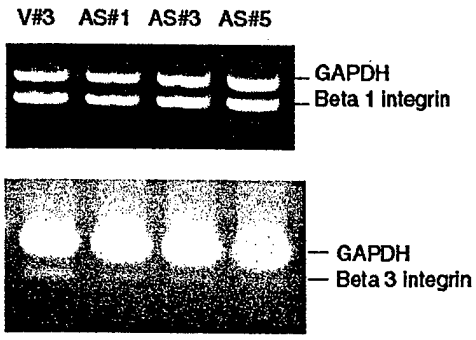
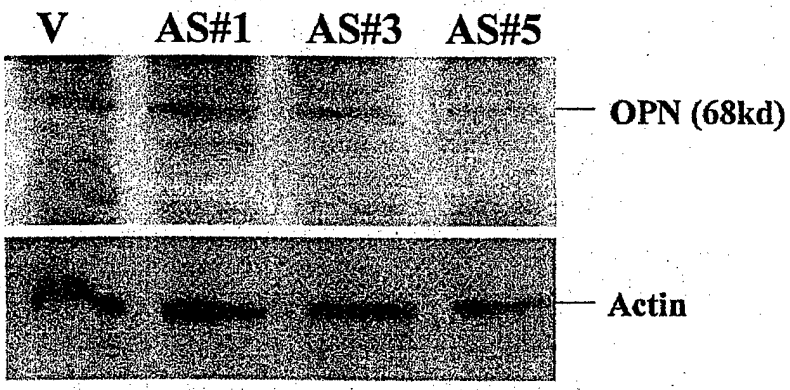


Figure 3: Expression of OPN protein in MDA-231-LC1 antisense clones by immunoblot of cell lysates



Levels of OPN protein normalized to actin levels:

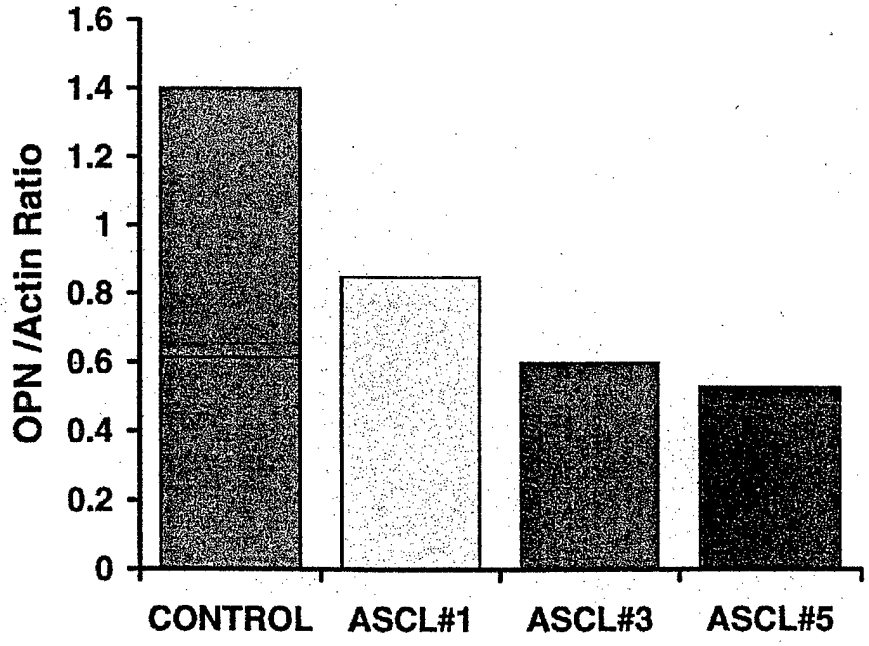


Figure 4a: Agarose colony formation

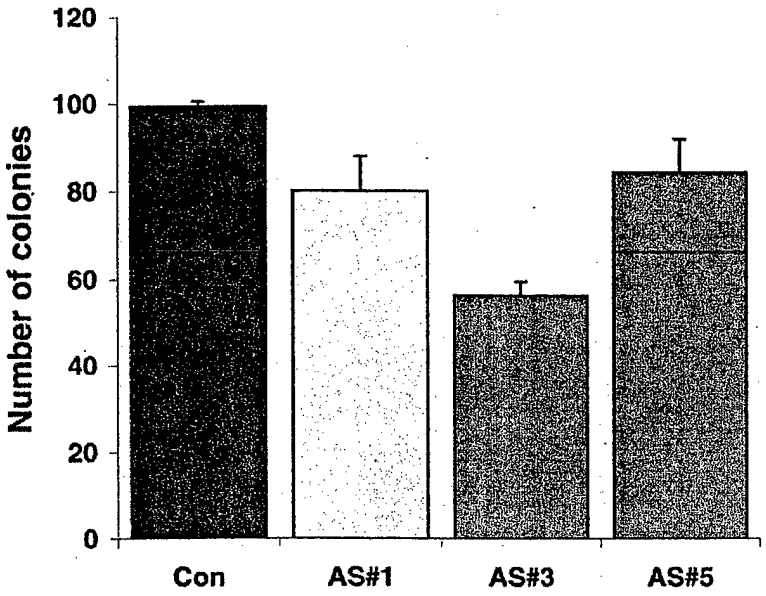


Figure 4b: Clonogenic growth